Overcoming Redundancy: an RNAi Enhancer Screen for Morphogenesis Genes in *C. elegans*

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ABSTRACT

JACOB MIGUEL SAWYER. Overcoming Redundancy: an RNAi Enhancer Screen for Morphogenesis Genes in *C. elegans*

(Under the direction of Bob Goldstein)

Morphogenesis is an important component of animal development. Genetic redundancy has been proposed to be common among morphogenesis genes, posing a challenge to the genetic dissection of morphogenesis mechanisms. Here, we present a screen designed to uncover redundant and partially redundant genes that function in an example of morphogenesis, gastrulation in *Caenorhabditis elegans*. We performed an RNAi enhancer screen in a gastrulation-sensitized double-mutant background, targeting genes likely to be expressed in gastrulating cells or their neighbors. Secondary screening was used to identify genes with detectable effects on gastrulation in both sensitized and non-sensitized backgrounds. By this method, we identified 16 new genes whose function is required for normal gastrulation in a non-sensitized background. We observed that for most of these genes, their closest known homologs were multiple other *C. elegans* genes, suggesting that some of these genes may have derived from rounds of relatively recent gene duplication events. We predict that such genes are more likely than single copy genes to comprise redundant or partially redundant gene families. We explored this prediction for one of the new genes. Our results confirmed that this gene and five close

relatives do indeed function partially redundantly with each other in gastrulation. Our results implicate new genes in *C. elegans* gastrulation, and show that an RNAi-based enhancer screen can be used as an efficient means to identify important but redundant or partially redundant developmental genes.

ACKNOWLEDGMENTS

When a person works on something for 6+ years, there are many people to thank along the way. I am pretty sure I have had a meaningful conversation with every Biology graduate student in my class and with most of the graduate students who have walked/paced the halls of Fordham in the time I was here. I think I was involved in recruitment every year, when Biology had our own recruitment, maybe even the year I entered to an extent, and so I met fellow students who didn't even go here and met the ones who were going to go here before they even joined. If I named everyone by name, this section of my thesis would be longer than the thesis itself. Just know that interaction, feedback, and conversation all matter to me.

My committee is awesome. I dare any grad student to tell me that their committee has been more supportive, more helpful, or more informative than mine. I want to especially thank Frank and Vicki for their mentorship of developmental biology and always giving me good advice when I needed it, Dave for being a friend and worm fountain of knowledge (perhaps you prefer walking Wormipedia), and Mark for being my second mentor and endless source of constructive criticism.

Bob and I have had our ups, downs, lefts, and rights. The highs and lows are self-explanatory, the lefts for Cambridge, Japan, and some small beach in South France, and the debates of rights versus wrongs, which by my count has Bob up by one (I still have time to eke this one out). I would especially like to thank him for teaching me that, "T-H-

E M-I-T-O-C-H-O-N-D-R-I-A I-S T-H-E P-O-W-E-R-H-O-U-S-E O-F T-H-E C-E-L-L" and that it is more than okay to be weird. I know I don't have the proper words to thank Bob for all he has done as a mentor or to point out how much I like him as a friend, but I will say that every PI should have a small, plastic siren whistle for when their students do something awesome.

The sixth floor of Fordham has been a great place to work. Beach weekend, when I have been able to attend, has been a blast. Playing "Villagers and Wolves" with Dyche Mullins, "CatchPhrase" with Ed Munro, or losing at poker to Xiaohu Wan are experiences I have to thank Kerry, Elaine, Ted, and Bob for. I have had great camaraderie with many of the folks on this floor. I specifically want to point out a few non-Goldstein labbers, in chronology, Jeff Molk was extremely friendly to me and helped a ton when I was TAing for Microbiology and I may be one of very few who have enjoyed a beer with him; Ryan O'Quinn still cracks me up with the driest delivered one-liners of all time; Julian Haase is the constant in an ever changing floor and we have enjoyed many a gaming and football conversation; Benjamin Harrison is easily one of the nicest guys I have ever met and has for some reason listened to a lot of my advice (as an aside, I passed on Molk's advice to Ben…and Steph…and Arthur…almost word-for-word); most people don't know that Tom Maresca and I had several "Biology at the Bar" nights, where we would present data to each other and discuss over way too many drinks, yet somehow always ended on politics. I want to also thank Jay Gatlin for being my only outlet for hunting stories and micro-forge maneuvering discussions. Big high five to Drew, your day in the sun will come soon enough. I also want to shout out to Jake, Ajit,

Dileep, David Bouck, and Daniela (and Christophe), who have all inspired or helped me in small, but important ways.

Big thanks to my second lab home, the Peifer lab. I just realized that I have known and been friends with over 19 undergrads, grad students, and post-docs in the lab. Let's see if I can do this chronologically: Don M. for teaching me that a baby strapped to your chest is no reason to slow you down in the lab; Don F. (and Anne) for being an awesome mentor, science nerd, and all around great guy; Tony H. (and Cheryl) for being super helpful, friendly, and Canadian; Meredith for long discussions on the craziness of PIs, Jeremiah (and Kelly), Dave R. (and Jenny), Erin J., Julie (and John), Jaime, Catarina (and Nuno), Nathan, Kelly (and Matt), Steph (and Linda), Doug, Mira, Rodrigo, Colleen (and Rich), John P. (and Caroline), and Ben R…and I am still probably forgetting somebody. Holy poop, you guys are awesome!

Of course, my first lab home is in 616 with my labbies. I remember going out to dinner with the Goldstein lab, when my Jess was rotating. I couldn't remember sitting at a table with so many funny, intelligent, and interesting people at the same time. OK, did I mention weird too. I have and do consider every member of the Goldstein lab my family. Seriously, I babysat Peyton for crying out loud. I think Jean-Claude, when I met him the first time, said it best, "Once a Goldsteinlabber, always a Goldsteinlabber," and it is true, although it is also funnier in a drunken, Quebecois accent. Every Post-Doc in the lab has helped me, made me a better scientist, and flat out been a friend. I seriously do not know where I would be without the mentorship of Dan for getting me started, Gidi for keeping me going, Jenny T. for being an unsung superstar, and Jessica (JSB) for helping me finish. Thank you guys so much! I also don't think hardly a day goes by, where I don't

think of former grad student lab mates: Nate (and Melvin) to know you is to love you and you really gave me some great early-years pep talks in your balcony office, Jen for making me sharpen my arguments and my science, Willow for educating me on the West and the strength of willpower, Erin (and John) for cracking me up every morning and being one of the few who danced with all my little dances, Minna (and Jarrod) for lots of great times and laughs, and Jessica (and Patrick) for always making me feel such a part of your family that I often got homesick for Cajun back-home cooking, storytelling, and family just by proximity (Jambalaya, crawfish pie, filet gumbo, …) . Adam, I still think we should go on the road with our comedy stylings, if nothing else we humor ourselves, but seriously, hang in there, someone as intelligent and hardworking as you will get rewarded in the end. Chris (and Jessica), in the short time I have known you, I already consider you part of the family, I wish you the best of luck, now if only I can convince you that ND will never contend for a national title again, ever. We have also had some pretty special undergrads: Shatil, Ty, Sapna, Shafali, Charlene, Aarti, Joe, Patty, and Terrence, but obviously a huge amount of appreciation goes to the two I mentored and had the honor of working with, Trudy Li and Stephanie Glass.

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(Cr(e^2)P^3!). Dan, thanks for being a mentor, friend, drinking partner, cheering section, and brother.

OK, speaking of brothers, my family has been very supportive of me through the years and I love them with all my heart. Edna, Pat, Johnathan, Maddie, Veronica, Paul, Madison, Tyler, Jason, Alison, Abby, and Jack, you guys are the world to me. I would not be anywhere without my parents and they have supported me emotionally when I have needed them for my whole life. I love you and thank you for always believing in me and letting me know how proud y'all are of me; I finally made it!!

I love you Jess. I can't wait to be back under the same roof and continue to spend the rest of our lives together. I must admit Drs. J. and J. Sawyer does have a nice ring to it.

PREFACE

What led me to grad school? How did I get to this point in my education? (Double rainbow. What does it all mean?) Actually, the rainbow comment is probably not that far off, I have been amazed by the natural world for far back as my memory will recall. The solar system, different cloud types, tides, sharks and fishes, snakes, rocks, and the list goes waaay on. I think I was pretty lucky to have lots of great teachers along the way, especially in math and science. Mrs. Long, Mr. Harrell, Mrs. George, Ms. Kinney, Mrs. Burgess, Mr. Powers, Mr. Haynes, Mrs. Miller, Mr. Olson, Ms. Kicklighter just to name a few off the top of my head. They all fueled my curiosity and kept me interested. My senior year the academic decathlon focus for SuperQuiz was health and biotechnology. So with a great biology teacher (Ms. Kicklighter) and an intense coach (Mr. Olson) our team crammed genetic disease info, DNA facts, and a history of the biological world into our brains…it was awesome. We were top 10 in the state of Texas.

Those early wonderful educators laid an excellent groundwork for me that I just about completely ignored as an undergraduate in college. I read whatever I felt like, sometimes that included textbooks and sometimes those textbooks were actually for a class I was taking, but that sometimes was less often than I would recommend. I didn't do horribly, but large lecture halls and general requirement classes bored me to tears. Luckily, I escaped my engineering major and switched to my new love biology, changing majors made me ineligible for the full ride scholarship I was on, so I moved to a different

University and had to retake some freshman classes, but it was worth it. I got interested again. Dr. Krause, Dr. Mailman, Dr. Hardin, and Dr. Wells taught me the details that I had missed concerning biochemistry, physiology, genetics, and human disease. I was excited again.

Long story, short, I went from undergrad lab dishwasher to Masters in 3 years. I moved to RTP and used my masters to do genetic testing for T and B cell lymphoma and Fragile X syndrome at LabCorp. In a lot of ways it was great doing blue-collar biology. Jess and I were able to settle down, life was good, but my brain was getting bored again. I apparently need to remain curious, I need to keep asking and answering questions. I knew I wanted to come back to grad school and I had audited a developmental biology course in Houston taught by Amy Sater, so, I also knew that I wanted to focus on a developmental question in a model organism.

I applied, they let me in, and life is good. Longer story, shorter, I have managed to exceed my expectations because I underestimated how much I had no idea about. Now the more you discover the more questions you have…awesome…perfect. I didn't burn out or go mental, which are both possibilities in grad school. I still like naming the types of clouds, rocks, sharks, double rainbows, etc., now I just include protein names, cell movements, and biological technology to that list, and yes, I am still in love with biology.

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Apical Constriction: A Cell Shape Change that Can Drive Morphogenesis

This chapter is adapted from a manuscript accepted to the journal Developmental Biology *(Sawyer et al., 2010). This was a collaborative effort, in that many lab members wrote drafts of sections that Bob Goldstein and I assembled and edited into a cohesive document. I was also responsible for the drafts for the Introduction, Drosophila gastrulation, and the Conclusions.*

ABSTRACT

Biologists have long recognized that dramatic bending of a cell sheet may be driven by even modest shrinking of the apical sides of cells. Cell shape changes and tissue movements like these are at the core of many of the morphogenetic movements that shape animal form during development, driving processes such as gastrulation, tube formation and neurulation. The mechanisms of such cell shape changes must integrate developmental patterning information in order to spatially and temporally control force production -- issues that touch on fundamental aspects of both cell and developmental biology and on birth defects research. How does developmental patterning regulate force-producing mechanisms, and what roles do such mechanisms play in development? Work

on apical constriction from multiple systems including *Drosophila*, *C. elegans*, sea urchin, *Xenopus*, chick and mouse has begun to illuminate these issues. Here, we review this effort to explore the diversity of mechanisms of apical constriction, the diversity of roles that apical constriction plays in development, and the common themes that emerge from comparing systems.

INTRODUCTION

Morphogenesis, the reorganization of cells and tissues into new forms, is an essential part of animal development. Cell and tissue reorganizations are driven by the forces that cells produce both internally and on neighboring cells. These forces are generally provided by the molecular motors that walk on intracellular polymers, the microfilaments and microtubules, or by polymerization and depolymerization of these polymers. How development controls these forces, to accomplish the morphogenetic movements that shape the final form of an animal, is a largely unanswered and yet central issue in developmental biology.

Biologists studying morphogenesis have recognized for over a hundred years that shrinking one side of a cell may result in a dramatic bending of a cell sheet. As early as 1902, Rhumbler proposed that constriction of the apical sides of cells may drive the bending of cell sheets in a variety of developmental systems (Figure 1.1) (Rhumbler, 1902). Physical modeling in the 1940s tested the feasibility of this hypothesis, with an epithelial sheet modeled using brass bars and rubber bands (Lewis, 1947). Lewis' model demonstrated that increased tension on one side – produced by Lewis adding more rubber bands to one side of his model – could result in bending.

Animals employ many distinct classes of morphogenetic movements. This review focuses on one class, apical constriction, or the active narrowing of cellular apices. Apical constriction occurs throughout the metazoa, and in many organisms, apical constriction first occurs at early stages of embryogenesis (Figure 1.2). This makes apical

constriction events valuable candidates for exploring the expected links between early patterning processes, such as cell fate specification or apico-basal cell polarization, and the mechanisms that produce force. Indeed, apical constriction is central to some key cases where we already understand at least an outline of the links between cell fate specification and the forces that drive morphogenesis, such as gastrulation in *Caenorhabditis elegans* and *Drosophila melanogaster* and vertebrate neural tube formation (Figure 1.3) (Chung and Andrew, 2008; Rohrschneider and Nance, 2009). Apical constriction also may underlie some of the other classes of morphogenetic movements, for example epithelial-to-mesenchymal transition and ingression (Keller and Davidson, 2004).

How then does apical constriction occur in cells? The most prevalent hypothesis is that apical constriction is driven by contraction of an apical meshwork of filamentous actin (F-actin) by the molecular motor myosin, but other mechanisms are plausible. Morphogenesis has been hypothesized to depend on redundant mechanisms to a large degree (Wieschaus, 1995). Do embryos use multiple, redundant mechanisms to drive apical constriction? Are there general rules by which apical constriction is regulated in diverse animal systems? What mechanical contexts are required for mechanisms to work, and how are these mechanical contexts established? We are also interested in the roles that apical constriction can play in development. Are common processes driven by apical constriction in diverse organisms? Are these processes conserved from ancestral animals of the past to groups of modern animals? These questions have additional importance because human neural tube closure depends on apical constriction, and improper neural tube closure is one of the most common human birth defects (Sadler, 2005). We focus on

these issues as we review some historical and well-studied examples of apical constriction.

Sea urchin gastrulation: multiple mechanisms may drive tissue bending

Perhaps surprisingly, given the large repertoire of classes of morphogenetic movements available to embryos, many organisms have evolved a role for apical constriction in gastrulation (Stern, 2004). In gastrulating sea urchin embryos, cells on the vegetal surface of the embryo become columnar, forming the vegetal plate. The surface of this plate bends inward, a process termed primary invagination (Figures 1.1 and 1.2). Primary invagination is accompanied by a number of other movements; here we discuss only the primary invagination, which has been proposed to be driven by apical constriction (for review see Davidson, 1995; Kominami and Takata, 2004).

The cells that undergo primary invagination form the archenteron, or future gut. Computer modeling suggests that apical constriction of cells in the vegetal plate could feasibly drive primary invagination, so long as the extracellular matrix can be deformed easily -- about as easily as the cells can be deformed (Davidson, 1995). In principle then, changes of individual cell shapes can drive tissue bending, although other mechanisms for bending a cell sheet are possible (Davidson, 1995). Forces generated within the vegetal plate are sufficient to drive tissue bending, as invagination can occur normally in a dissected vegetal plate (Moore and Burt, 1939; Ettensohn, 1984). The cells proposed to undergo apical constriction have bands of actin microfilaments associated with apical adherens junctions and also spanning across the inside of each cell's apical surface, as might be expected in cells undergoing apical constriction. But microfilaments are also enriched apically in cells that do not undergo such shape changes. Hence the presence of

such an apical microfilament network does not necessarily indicate that it will bend a cell sheet (Ettensohn, 1984).

In certain species of sea urchin, a ring of cells along the edges of the vegetal plate has been recognized to undergo more pronounced apical constriction, as judged by scanning electron micrographs (Nakajima and Burke, 1996, Kimberly and Hardin, 1998, Figure 1.2). Cells in this ring have been referred to as bottle cells, a term coined by Ruffini (1907) for amphibian embryonic cells that are shaped like bottles, with dramatically constricted apical sides and enlarged basolateral areas. Bottle cells in sea urchin embryos have a greater enrichment of apical arrays of F-actin than do other cells in the vegetal plate (Nakajima and Burke, 1996). Laser ablation of bottle cells interferes with normal invagination, whereas laser ablation of neighboring cells does not (Kimberly and Hardin, 1998), consistent with the notion that apical constriction may drive primary invagination. RhoA is required for the initiation of primary invagination (Beane et al., 2006), as it is for apical constriction and resulting tissue bending in other systems discussed below. How are specific cells driven to apically constrict during primary invagination? This is not yet clear, although calcium signaling (Nakajima and Burke, 1996), Wnt/Frizzled signaling (Croce et al., 2006), a transcriptional gene regulatory network (Davidson et al., 2002; Wu et al., 2008), and FGF signaling (Röttinger et al., 2008) have all been implicated in regulating primary invagination. The links between these regulators and RhoA activity have yet to be explored.

One key result is at odds with the model that actomyosin-dependent apical constriction is the key driver of primary invagination: cytochalasin treatment, which should depolymerize F-actin networks, fails to fully disrupt primary invagination in sea

urchins (Lane et al., 1993). This result suggests the possibility that other mechanisms may provide force, either alone or redundantly with actin-based mechanisms. Interestingly, among the mechanisms proposed to drive apical constriction and tissue bending in sea urchins during primary invagination is one in which vegetal plate cells secrete extracellular matrix components into a multi-layered structure, in a calcium regulated manner (Lane et al., 1993). In this model, later-deposited matrix, secreted into a layer between the cells and the earlier layers of matrix, swells as it hydrates, driving bending of the matrix and hence the attached epithelial sheet. This is similar to the way in which the thermal expansion of a layer of metal in a thermostat's bimetallic strip can bend the entire strip. In Lane et al.'s model, the proposed source of force is extracellular, driving cell shape changes by bending of the matrix, rather than mediated by intrinsic cell shape changes, an interesting departure from traditional models. As an experimental model, sea urchin primary invagination leaves a variety of possible mechanisms for tissue shape change and some valuable tools for dissecting the contributions to forces made by each.

Bottle cells in *Xenopus* gastrulation: roles for microfilaments and microtubules

The amphibian archenteron also includes bottle cells at the site where invagination begins (Holtfreter, 1943). Early embryologists believed that amphibian bottle cells functioned in gastrulation because of the cells' unique shapes (Figure 1.2). Rhumbler (1902) suggested the possibility that these cells were actively migrating toward the interior of the embryo. Experiments by Holtfreter were consistent with this hypothesis, as isolated bottle cells could stretch in a polarized manner on a glass substrate, similar to migrating cells (Holtfreter, 1944). While no live imaging evidence exists for the active migration of these bottle cells *in vivo*, vital dye tracings demonstrate

that these cells do migrate to the interior of the embryo in *Ambystoma mexicanum* (Lundmark, 1986). In addition, cell tracing experiments in which labeled bottle cells from Xenopus laevis were grafted into unlabeled host embryos demonstrate that bottle cells spread out and form the anterior of the archenteron (Hardin and Keller, 1988).

Xenopus laevis bottle cells (Figure 1.4) are a potentially valuable model for studying mechanisms of cell shape change in morphogenesis, as the cells are large and readily treated with inhibitors. These cells can be manipulated in culture much as sea urchin cells can be, and the potential exists to identify key molecular players using genetic screens in the model frog *Xenopus tropicalis*. Blastopore initiation begins and proceeds on schedule in explants that include the bottle cells (Hardin and Keller, 1988; Lee and Harland, 2007). When bottle cells are removed from *X. laevis* embryos, a truncated archenteron still forms, and involution of the mesoderm cells still occurs, but archenteron length is compromised (Keller, 1981). Therefore, bottle cells appear to initiate blastopore formation and to contribute to the full extension of the archenteron in *X. laevis*.

A number of distinct mechanisms control cell shape in *X. laevis* bottle cells. *In vivo*, the apical surfaces of these cells shrink while the apicobasal sides lengthen. Isolated, cultured bottle cells contract uniformly around the entire cell surface, suggesting that contraction is an intrinsic behavior but that the apicobasal elongation seen *in vivo* depends on contact with surrounding cells (Hardin and Keller, 1988). This likely reflects a cellular mechanism that distinguishes the basolateral and apical sides of bottle cells, or surfaces contacting other cells and free surfaces, perhaps similar to a mechanism that has been outlined in *C. elegans*, discussed below. F-actin and activated myosin accumulate at

the apical surfaces of bottle cells just before the apical surfaces narrow, consistent with a role for F-actin and myosin in apical constriction (Lee and Harland, 2007). Furthermore, pharmacological inhibitors of F-actin or myosin demonstrate that they are both required for bottle cell formation. Interestingly, treatment with a microtuble depolymerizing drug, nocodazole, prevents full apical constriction of bottle cells and invagination without affecting apicobasal cell lengthening, and without apparent effects on F-actin or activated myosin distribution (Lee and Harland, 2007). This result suggests that microtubules may have an as yet undefined role in apical constriction in *Xenopus* bottle cells.

C. elegans gastrulation: cell manipulations and genetics meet to identify key regulators

Unlike gastrulation in sea urchins or *Xenopus*, where entire cell sheets are internalized, gastrulation in *C. elegans* involves the internalization of many cells or groups of cells at distinct times. *C. elegans* gastrulation begins at the 26-cell stage when two endodermal precursor cells move from the perimeter to the inside of the embryo (Figure 1.5). This event is followed later by internalization of mesoderm and germline precursors (Sulston et al., 1983; Nance and Priess, 2002). Internalization of the endodermal precursors has been most thoroughly studied and is the focus of our discussion here.

Cell movements associated with *C. elegans* gastrulation can occur *in vitro*, allowing mechanisms to be explored by cell manipulation experiments as in sea urchins and *Xenopus* (Lee and Goldstein, 2003). One revealing finding from such studies is that very few cells are required for the movements of *C. elegans* gastrulation to occur: even a line of embryonic cells in culture arranged in single file will fold at the time of

gastrulation (Lee and Goldstein, 2003). This makes clear that mechanisms requiring large numbers of cells to work in concert, such as multicellular purse string mechanisms, are not essential for cell movements in *C. elegans* gastrulation. Some of the strengths of this system lie in the ability to combine such manipulations with live cell microscopy and genetics, and to study mechanisms of morphogenesis at the level of individual cells, in a developmental system where spatial patterning is so thoroughly studied.

Apical constriction plays a key role in *C. elegans* gastrulation. Just before endodermal precursor cells internalize, the cell surface that faces the perimeter of the embryo on each of these cells (the apical surface) flattens, and myosin II becomes enriched at this surface (Nance and Priess, 2002). Although the apical surfaces become smaller until they disappear at the time of cell internalization, these cells do not become noticeably bottle-shaped. Contraction of apical cell surfaces was revealed by tracking the movements of fluorescent, microscopic beads placed on the surfaces of the endodermal precursor cells (Lee and Goldstein, 2003). The observed surface movements exclude the possibility that shrinking of the apical surface reflects only a flow of apical surface to lateral positions -- a possibility that is difficult to exclude in many systems. Myosin has been implicated in driving apical constriction because pharmacological inhibitors of myosin activity prevent the endodermal precursors from internalizing (Lee and Goldstein, 2003). In addition, apical myosin becomes activated near the time that gastrulation begins: apically-localized myosin regulatory light chain is phosphorylated at a residue that in other systems unkinks myosin heavy chains, allowing myosin complexes to bundle into bipolar filaments, which can bind to and walk on actin filaments (Lee et al., 2006; Somlyo and Somlyo, 2003). These results suggest that local activation of myosin

shrinks the apical actin mesh. Actin architecture is likely to be important as well. Indeed, the Arp2/3 actin-nucleating complex has been reported to localize to the cell cortex in gastrulating embryos, and depletion of this complex results in failure of endodermal precursor cells to internalize on schedule (Severson et al. 2002, Roh-Johnson and Goldstein, in press).

Do neighboring cells contribute to internalization of the endoderm precursors? When neighboring cells were removed and reassociated with endodermal precursor cells in various orientations, the neighboring cells still moved in a direction consistent with the hypothesis that apical constriction in endodermal precursors drives the movement of the neighboring cells, suggesting that neighboring cell polarity is not important for the bulk of their movement (Lee and Goldstein, 2003). However, short, actin-rich extensions form on three of the six neighboring cells of the ring that closes beneath the endoderm precursors, and Arp2/3-depleted embryos that fail to gastrulate also fail to produce these extensions, raising the possibility that the extensions might contribute to completion of endodermal internalization *in vivo* (Nance and Priess, 2002; Roh-Johnson and Goldstein, in press).

C. elegans genetics has identified multiple regulatory inputs that are important for gastrulation, including inputs that specify which cells should enrich myosin to one side, inputs that specify to which side of a cell this enrichment should occur, as well as a signaling input that directs activation of myosin. Cell fate specification genes including genes encoding endodermal GATA factors are necessary for early cell internalization, and embryos with ectopically specified endoderm have ectopic early cell internalization, suggesting that endoderm fate is both necessary and sufficient for early cell

internalization (Lee et al., 2006). One aspect of endodermal cell fate is a gap phase uniquely introduced to the cell cycle of endodermal progenitors one cell cycle after the endoderm precursor cell is born, which is near the time of cell internalization (Sulston, 1983; Edgar and McGhee, 1988). This pause is required for internalization, possibly because it delays a reorganization of the actomyosin cytoskeleton that normally accompanies cell division (Lee et al., 2006; Oegema and Hyman, 2006).

For the endodermal precursor cells to accumulate myosin near their apical surfaces, an apical surface must be established. PAR proteins function in anteroposterior polarization of the embryo first, and are known to become apicobasally polarized later, starting at the four cell stage (see Goldstein and Macara 2007 for review). To test whether PAR proteins function in apicobasal polarization, Nance and colleagues devised a clever method for degrading the polarity proteins PAR-3 or PAR-6 specifically in somatic cells, adding a motif from another protein that becomes degraded in somatic cells. They demonstrated in this way that PAR-3 and PAR-6 are required for apical flattening, apical myosin enrichment, and timely cell internalization (Nance et al., 2003). Elegant cell manipulation experiments revealed that these PAR proteins' localization depends on where cells contact each other: only contact-free membranes accumulate apical PAR proteins, establishing an apical domain at the contact-free surface (Nance et al., 2003). Myosin later accumulates at apical domains, and this is dependent on apical PAR proteins (Nance et al., 2003). Once myosin becomes enriched apically, it becomes activated downstream of a Wnt-Frizzled-Dishevelled signaling pathway that causes regulatory light chain phosphorylation, through an unidentified kinase (Lee et al., 2006, Figure 1.3).

These results paint the outlines of a potentially generalizable mechanism for cell internalization by apical constriction: Among cells that polarize PAR proteins apicobasally, the cells with the right cell fate specification machinery enrich myosin where the apical PAR proteins become localized -- at contact-free surfaces. Activation of myosin can then result in shrinking the myosin-enriched, contact-free surfaces of any such cells, pulling neighboring cells across the free surfaces and, as a result, displacing the apically constricting cells toward the interior. The ability to shrink any exterior surface of specific cells could, in theory, make it possible for a cell to internalize regardless of which specific surfaces initially contact other cells.

How then do certain PAR proteins become enriched only apically in response to cell contacts? Anderson et al. (2008) screened for genes required for cell contact-dependent PAR protein localization and identified a key intermediate, a RhoGAP domain-containing protein, PAC-1. PAC-1 localizes to the cell cortex at cell-cell contact zones, where it has been proposed to inactivate CDC-42 at these zones, potentially restricting the active form of CDC-42 to contact-free cell surfaces. Active CDC-42 interacts with a semi-CRIB domain in PAR-6, and through this interaction is thought to establish apical localization of PAR-6 and PAR-6 complex members in these cells. PAC-1 localization to contact zones is therefore the earliest known step in recognizing contact zones as unique, spatial information that is critical to PAR protein and myosin localization. How PAC-1 becomes localized to contact zones is an interesting topic for future study.

Drosophila melanogaster gastrulation: links from cell fate to the cytoskeletal machinery that provides force

The initiation of morphogenesis in fruit flies begins with the internalization of the future mesoderm at the ventral furrow, forming a tube in the interior of the embryo (Leptin and Grunewald 1990). Ventral furrow formation is perhaps the most well studied example of apical constriction, and the cellular shape changes that occur have been thoroughly described. First, a stripe of cells 18 cells wide and 60 cells long, spanning most of the embryo's ventral midline (6% to 86% egg length), begins to apically flatten. Within this stripe, after flattening, cells of the midventral domain, 12 cells in width, begin to reduce the diameter of their apical surfaces. As these ventral midline cells apically constrict, small blebs form on the apical membrane surfaces, possibly aiding in the reduction of apical surface area (Turner and Mahowald 1977; Costa et al., 1994). Other rearrangements can be seen as ventral midline cells' apical surfaces begin to shrink. For instance, cytoplasm and the nuclei of the midline cells shift basally (Leptin and Grunewald, 1990). The cells also elongate along their apicobasal axes up to 1.7 times their original lengths, and then expand their basal surfaces (Leptin and Grunewald, 1990; Sweeton et al., 1991). After reaching their maximum lengths, the ventral furrow cells begin to shorten back to their original length, while remaining constricted apically Sweeton et al., 1991). Shortening of each cell results in a wedge shape that may help to move the ventral furrow beneath the epidermis (Costa et al., 1993). Completing the process, the lateral epidermis covers the tube of mesoderm, pinching it off from the overlying ectoderm (Figure 1.6) (Poulson, 1950; Sonnenblick, 1950; Leptin and Grunewald, 1990).

The power of *Drosophila* as a genetic model system is illustrated by a pathway that spans from cell fate specification to the force-producing mechanisms that drive

apical constriction in gastrulation (Figure 1.3). Determination of mesodermal fate is governed by the maternal transcription factor Dorsal, which activates ventral expression of the zygotic transcription factors Snail and Twist (Simpson 1983; Nusslein-Volhard et al., 1984; Thisse et al., 1987). Loss of Snail and Twist prevents furrow invagination (Leptin and Grunewald, 1991; Sweeton et al., 1991) and expands lateral cell fates toward the ventral midline (Costa et al., 1993). No targets of Snail that function in ventral furrow formation have been identified thus far, but targets of Twist with specific roles in cell shape changes have been identified. Loss of the Twist target Folded Gastrulation (Fog), a secreted protein, leads to uncoordinated constriction during gastrulation, in which cell shape changes are initiated in many cells at the correct time, but some cells fail to undergo apical constriction (Sweeton et al., 1991; Costa et al., 1994). Loss of the Gα protein Concertina (Cta) results in a similar phenotype, and Cta acts genetically downstream of Fog (Morize et al., 1998). Presumably, there is a G-coupled receptor that links Fog signaling to Cta; to date, it remains unknown. Interestingly, the secretion of Fog protein is apically polarized, and Fog is both necessary and sufficient to target myosin to the apical side of the cell (Dawes-Hoang et al., 2005). A second downstream target of Twist, the transmembrane protein T48, functions in parallel to Fog-Cta signaling (Kolsch et al., 2007). Loss of both Cta and T48, each of which have weak gastrulation phenotypes, i.e. uncoordinated apical constriction, results in failure to make a furrow, suggesting that these two pathways act partially redundantly (Kolsch et al., 2007).

How do these proteins result in force generation? Both the Fog-Cta and T48 pathways converge on the localization of RhoGEF2, a regulator of the Rho family GTPases (Barrett et al., 1997; Rogers et al., 2004; Kolsch et al., 2007). In RhoGEF2

mutants, apical constriction does not occur and the ventral furrow never forms, as in the Cta and T48 double mutants (Barrett et al., 1997; Hacker and Perrimon, 1998, Kolsch et al., 2007). Both T48, which directly binds RhoGEF, and Cta mildly affect the localization of RhoGEF2, but if both proteins are absent, RhoGEF2 does not become apically localized (Kolsch et al., 2007). Disruption of RhoGEF2 in ventral furrow cells disrupts myosin accumulation and localization, and the cells are unable to constrict (Nikolaidou and Barrett, 2004). A similar, albeit not as dramatic, myosin mislocalization phenotype has been observed in *cta* mutants (Nikolaidou and Barrett, 2004; Fox and Peifer, 2007). RhoGEF2 most likely functions through activation of Rho1, since dominant-negative Rho1 results in ventral furrow defects (Barrett et al., 1997). Both the myosin II regulatory light chain Spaghetti Squash (Sqh) and the myosin II heavy chain Zipper (Zip) become relocalized from the basal side of the cell to the apical side, along with RhoGEF2, which fly cell culture has demonstrated to be bound to microtubule tips via EB1 (Rogers et al., 2004; Nikolaidou and Barrett, 2004). Interestingly, activated Cta is also required to unload RhoGEF2 from microtubule tips to the plasma membrane (Rogers et al., 2004), by an unknown mechanism. Together, these findings build a pathway that links cell fate through signaling components to cytoskeletal regulators (Figure 1.3).

If myosin activation and localization is key, then a specific F-actin organization would be predicted to be important as well. Early work proposed that the apical F-actin cytoskeleton was required for internalization of the ventral furrow cells (Young et al., 1991). Further work has explored just how the actomyosin meshwork must be organized and dynamically regulated for apical constriction to occur. Abelson (Abl), a non-receptor tyrosine kinase, is required for the correct localization of F-actin within the ventral

furrow cells (Fox and Peifer, 2007). *abl* mutants also have uncoordinated apical constriction at the ventral furrow, like *fog* and *cta* mutants. *cta* mutants do not have mislocalized F-actin, but *RhoGEF2* mutants do, suggesting that Abl functions in parallel to RhoGEF2 in regulation of F-actin localization. Abl targets F-actin organization through a known target, the actin regulator Enabled (Ena). Within the ventral furrow cells, Abl regulates Ena localization, restricting it from the apical end (Fox and Peifer, 2007). One other actin regulator implicated in ventral furrow formation is the formin protein Diaphanous (Dia), which along with Rho kinases is a Rho effector, suggesting that actin regulation and myosin regulation might be coordinated by multiple Rho effectors upon Rho activation (Homem and Peifer, 2008). RhoGEF2 and Dia are also necessary during cellularization for the correct assembly of actin filaments that are required for the proper infolding of the plasma membrane (Grosshans et al., 2005).

The actomyosin network of each ventral furrow cell spans beneath the entire apical surface of the cell and also forms circumferential belts at the apical boundaries with neighboring cells, at adherens junctions (Costa et al., 1993; Martin et al., 2008). Martin et al. have proposed that apical constriction is driven by pulsed coalescences of the actomyosin meshwork across the entire apical surface, rather than being driven by the circumferential belts of actin (Martin et al., 2008). Each pulse of actomyosin coalescence appears to shrink the apical surface, and, in general, each coalescence does not retreat, suggesting the existence of a ratchet-like mechanism limiting expansion of the apical cytoskeleton after each constriction. Interestingly, differential roles for the mesoderm specification proteins Snail and Twist were found: Snail promotes contraction, whereas Twist is necessary to prevent relaxation after each contraction, suggesting that the

proposed ratchet involves one or more Twist targets (Martin et al., 2008). Interestingly, invagination of the furrow can be rescued in *snail* mutants by mechanical deformation of the mesodermal cells, as long as the Twist target Fog is still present (Pouille et al., 2009). This is consistent with the hypothesis that Snail's role is in producing mechanical deformation, or contraction, and that Twist-dependent ratcheting is important to maintain contracted states. Mechanical deformation can induce Twist expression, suggesting an intriguing feedback loop between gene expression and deformation of cells that may serve to coordinate cells and increase the robustness of the system (Farge 2003; Desprat et al., 2008). Such feedback between gene expression and deformation of cells has not been explored similarly in other systems for apical constriction, to our knowledge.

These coordinated cell shape changes occur within a tissue in which cells are mechanically coupled. The actomyosin coalescences that ratchet the apical surfaces together are attached to adherens junctions at discrete sites. As each cell pulls the plasma membrane inward, connected to its neighbors, the result is the coordinated apical constriction across the epithelial sheet (Martin et al., 2008). In fact, if the adherens junctions are disrupted, myosin II coalesces into a ball detached from the cell contact zones, in the apex of each cell, and cells fail to change shape (Dawes-Hoang et al., 2005). Therefore, the adherens junctions provide mechanical links between the apical actomyosin network and the plasma membranes at cell contacts. Interestingly, apical localization of adherens junction components depends on Bazooka, a homolog of *C. elegans* PAR-3. PAR-3 functions in *C. elegans* gastrulation as well, but in apical enrichment of myosin rather than of adherens junction complex members, suggesting that

similar apicobasal cell polarity inputs can function differently in the two systems (Müller and Wieschaus, 1996; Nance et al., 2003; Harris and Peifer, 2004).

Although the F-actin meshwork is connected to the adherens junctions, this connection is not thought to be a direct link from F-actin to the adherens junction proteins alpha catenin, beta catenin, and cadherin (Weis and Nelson, 2005). Instead, other adhesion proteins may provide a mechanical link to the adherens junction complex. For instance in fly gastrulation, the afadin homolog Canoe, a scaffolding protein, aids in connecting the adherens junction to F-actin (Sawyer et al., 2009). The GTPase Rap1 regulates this interaction, and in both Rap1 and Canoe mutants, actomyosin coalesces into apical balls (Sawyer et al., 2009).

The internalization of the endoderm of the posterior midgut is also completed by an apical actomyosin constriction. At the posterior pole, a population of cells under and near the pole cells forms a cup-shaped invagination (Costa et al., 1993). Similar to ventral furrow formation, formation of the posterior midgut invagination begins with apical flattening (Sweeton et al., 1991). As the apices constrict, the nuclei move basally, and the cells increase their apicobasal lengths and expand their basal widths (Turner and Mahowald, 1977; Sweeton et al., 1991; Costa et al., 1993). Many of the proteins used in ventral furrow formation also drive posterior midgut invagination, but there are some interesting differences that demonstrate that the redundancy of mechanisms can vary between tissues. In the ventral furrow, mutations in Fog and Cta only partially disrupt invagination, whereas in posterior midgut invagination, loss of either of these two proteins completely prevents invagination (Parks and Wieschaus, 1991; Sweeton et al., 1991). RhoGEF2 is also required for posterior midgut invagination, again most likely via

Rho1 (Barrett et al., 1997), and cytoplasmic myosin is localized apically in posterior midgut cells (Young et al., 1991). Unlike ventral furrow formation, two other morphogenetic movements besides apical constriction contribute to posterior midgut invagination: dorsal retraction and germband elongation (Costa et al., 1993). Also, Canoe does not seem to be essential for posterior midgut invagination, though Rap1 may still play a role (Sawyer et al., 2009). The model developed for *Drosophila* gastrulation, in both ventral furrow formation and posterior midgut invagination, has close parallels in other morphogenetic events in *Drosophila*, as discussed below.

Drosophila eye morphogenetic furrow: a traveling wave of cell shape changes

Patterning of the *Drosophila* eye is accompanied by a wave of apical constriction (Figure 1.7) that passes across a sheet of epithelial cells, the eye imaginal disc. This wave is driven by a wave of cell-cell signaling events. In the posterior margin of the eye imaginal disc, some of the epithelial cells differentiate as photoreceptor neurons. Once differentiated, these cells secrete a Hedgehog ligand, which activates a signaling pathway in the anterior neighboring cells (Heberlein et al., 1993). Responding to this pathway, the latter cells enter cell cycle arrest, followed by apical constriction, resulting in the formation of a dorso-ventral groove known as the morphogenetic furrow (Ready et al., 1976; Tomlinson, 1985). Most of the cells in the furrow soon re-enter the cell cycle, relax, and resurface. Some of the cells undergo cell shape changes and differentiate, becoming the next group of photoreceptors. These newly differentiated cells then induce a new wave of Hedgehog-dependent furrow induction, received by the next row of anterior epithelial neighbors. Thus, over the course of a few hours, there is a wave of

morphogenetic furrow progression from posterior to anterior, followed by a synchronized process of neuronal differentiation (Ready et al., 1976).

Morphogenetic furrow progression in the eye imaginal disc probably contributes to the planar polarity of the disc epithelium (Chanut and Heberlein, 1995), but it is not completely clear whether apical constriction is needed for cells to properly differentiate. One hypothesis yet to be tested is that apical constriction may result in accumulation of receptors, such as Notch or EGFR, on the apical membrane, allowing cell communication and successive rounds of differentiation to take place (Wolff and Ready, 1991).

Apical constriction in the eye furrow involves some of the same proteins used by ventral furrow cells to achieve apical constriction during gastrulation. F-actin and myosin II accumulate at the apical cortex, and activation of myosin II allows the actomyosin apical network to contract, promoting constriction. Myosin activation is mediated through phosphorylation of its regulatory light chains by a Rho-dependent kinase (ROK) and negatively regulated by a myosin regulatory light chain phosphatase (Lee and Treisman, 2004; Corrigall et al., 2007; Escudero et al., 2007). Although ROK activates myosin II, there are likely to be other inputs to myosin activation, as phosphomimetic myosin but not constitutively active ROK is sufficient for formation of ectopic morphogenetic furrows (Corrigall et al., 2007; Escudero et al., 2007). Microtubules also play a role here, in normal actin organization: apical accumulation of the actomyosin network is accompained by apical accumulation of stabilized microtubules, and severing of microtubules by expression of the severing protein Spastin results in failure of the cells to apically constrict (Corrigall et al., 2007).

One significant difference between the eye morphogenetic furrow and the ventral furrow lies in the regulation of the actomyosin network. Unlike the ventral furrow, genetic evidence suggests that there is no role for RhoGEF2 in regulating Rho1 to promote apical constriction in the eye morphogenetic furrow (Corrigall et al., 2007). In addition, there is no genetic evidence to date for regulation of F-actin reorganization by Abl or Ena, which function in ventral furrow formation (Corrigall et al., 2007; Fox and Peifer, 2007). There is at least one actin regulator that functions in both systems: the formin Diaphanous (Dia) is needed for apical accumulation of F-actin and myosin and for apical constriction to take place in both the eye morphogenetic furrow and the ventral furrow (Grosshans et al., 2005; Corrigall et al., 2007; Homem and Peifer, 2008). The stories diverge significantly when it comes to the molecular pathways that govern cell fates, determining which cells will apically constrict. Unlike mesodermal cells in the ventral furrow, Twist and Snail do not have a role in eye patterning. Instead, Hedgehog signaling acts with the BMP homolog Decapentaplegic (Dpp), regulating microtubule stabilization, F-actin apical accumulation, myosin regulatory light chain phosphorylation, and Cad86C expression, which in concert lead to apical constriction and formation of the morphogenetic furrow (Corrigall et al., 2007; Schlichting and Dahmann, 2008; Vrailas and Moses, 2006; Escudero et al., 2007). Hence, different fate regulators and intracellular signals can function upstream of common cytoskeletal players to drive apical constriction in different tissues.

Drosophila trachea and salivary glands and the chick inner ear: formation of tubes and vesicles

Tube formation is another morphogenetic process that involves apical constriction. Two well-studied cases, the tracheal tubes and the salivary glands in *Drosophila*, give us some insights into the cellular and molecular mechanisms that govern apical constriction during tube formation. Tube formation in both cases starts with cells at the embryonic surface apically constricting and invaginating (Figure 1.8). Further branching and elongation by cell migration and convergent extension results in tubular structures with diverse functions (Myat, 2005).

The tracheal system is an interconnected network of branched epithelial tubes, responsible for gas transport. Trachea form from clusters of cells, each called a tracheal placode (Myat, 2005). During embryogenesis, ten placodes invaginate on each side of the *Drosophila* embryo. The onset of each of these invaginations is marked by apical constriction of about six cells in each placode. As the invagination deepens, it appears to turn (Figure 1.8), resulting in the formation of a finger-like invagination turned dorsally below the embryo surface (Brodu and Casanova, 2006). As in other systems, apical constriction is preceded by an accumulation of F-actin and myosin II at the apical cortex of each constricting cell (Brodu and Casanova, 2006). Myosin and F-actin enrichment are interdependent, as mutant isoforms of myosin that cannot bind to actin fail to localize apically and, conversely, F-actin is not apically enriched in myosin mutants (Brodu and Casanova, 2006).

Tracheal apical constriction is regulated by upstream patterning genes. Cell fate is governed by the Trachealess bHLH/PAS transcription factor. Trachealess expression defines a region of cells that will later invaginate, positively regulating actomyosin accumulation and apical constriction by activating the EGFR signaling pathway through

transcription of the EGF regulator Rhomboid (Affolter and Caussinus, 2008; Brodu and Casanova 2006; Nishimura et al., 2006). This regulation of actomyosin accumulation is mediated by Rho activity, by the function of the RhoGAP Crossveinless and its downstream target Rho1 (Brodu and Casanova, 2006).

The Drosophila salivary glands originate from two ventrolateral, ectodermal placodes (Myat and Andrew, 2000). The cells found in these placodes apically constrict and invaginate in a sequential manner during embryogenesis, starting from the dorsal-posterior portion of the placodes and progressing to other regions. F-actin and myosin accumulate at the apical cortex of constricting cells (Nikolaidou and Barrett 2004), and ROK-dependent phosphorylation of myosin contributes to apical constriction (Xu et al., 2008). Similar to the eye morphogenetic furrow, ROK mutants show only partial defects, suggesting that other kinases act redundantly with ROK to activate myosin contraction. ROK is again regulated here by Fog, Cta, RhoGEF2 and Rho1 (Nikolaidou and Barrett, 2004, Xu et al., 2008), as well as by 18wheeler, a Toll receptor protein that promotes Rho signaling, possibly through inhibition of the RhoGAP crossveinless (Nikolaidou and Barrett, 2007; Kolesnikov and Beckendorf, 2007). Interestingly, comparing phalloidin staining to anti-actin labeling in these cells shows that while total actin is evenly distributed along the cortex, filamentous actin is enriched specifically at the apical domains of the constricting cells. The kinase Tec29 maintains the imbalance between filamentous and monomeric actin (Chandrasekaran and Beckendorf, 2005).

During formation of the chick inner ear, otic ectodermal cells apically constrict, forming a vesicular otocyst within the head mesenchyme (Meier, 1978; Alvarez and Navascues, 1990). Elegant experiments involving extracted chicken tissues treated with

various compounds suggested that the invagination of the ectodermal cells does not rely solely on cell-autonomous apical constriction, but probably involves forces exerted from the surrounding mesenchyme (Hilfer et al., 1989). Interestingly, apical constriction of the otic ectodermal cells involves an unconventional mechanism for F-actin localization. Instead of co-localizing with F-actin at the apical cell cortex, phosphorylated myosin accumulates at the basal domains of the cells, and its activity leads to local F-actin depletion and the resulting enrichment of F-actin at the apical domain (Sai and Ladher, 2008). Myosin-dependent F-actin depletion has been shown previously both *in vitro* (Haviv et al., 2008) and *in vivo* (Medeiros et al., 2006), but the mechanism(s) behind this are not yet clear. It is interesting to note that reciprocal localization of myosin II and F-actin is also detected during early neural tube formation (Sai and Ladher, 2008).

Drosophila dorsal closure and *Xenopus* wound healing: apical constriction contributes to sealing openings

Dorsal closure occurs halfway through *Drosophila* embryogenesis, when a pair of lateral epithelial sheets migrate from each side of the embryo, closing a hole on the dorsal side (Campos-Ortega and Hartenstein, 1985). Prior to this, the hole is transiently filled by an extra-embryonic epithelium, the amnioserosa.

The forces that drive dorsal closure have been dissected extensively by examining movements that occur as immediate responses to cutting specific tissues using a laser. Forces produced by both the amnioserosal cells and the cells of the epidermis regulate dorsal closure. The leading edge of the advancing epidermis forms a supracellular actin cable whose contraction contributes forces for dorsal closure (Kiehart et al., 2000*)*. Initially, it was thought that the lateral epidermis migrated over passive amnioserosal

cells (Campos-Ortega and Hartenstein, 1985). However, transmission electron microscopy has revealed that the amnioserosal cells shift from a squamous to a columnar cell shape, constricting their apical surfaces during dorsal closure (Rugendorff et al., 1994). Amnioserosal cells also drop out of the plane of the surface of the embryo (Kiehart et al., 2000) by apical constriction coupled to apoptosis (Toyoma et al., 2008). When amnioserosal cells were severed by laser cutting, or selectively killed by expressing ricin in these cells, dorsal closure was impaired (Kiehart et al., 2002; Scuderi and Letsou, 2005). These results indicate that the amnioserosal cells contract, contributing to closure forces.

The amnioserosal cell forces can act redundantly with the supracellular purse string in producing forces that drive dorsal closure (Kiehart et al., 2000; Hutson et al., 2003). When amnioserosal cells are cut with a laser, the leading edge recoils, but ultimately dorsal closure completes. The same is true for the leading edge – when the leading edge is severed, dorsal closure still completes. However, when both the amnioserosal cells and supracellular purse string are severed, dorsal closure is impaired (Kiehart et al., 2000), indicating that either tissue is able to compensate for cuts in the other. The recoil seen after cutting either tissue indicates that both tissues are under tension. Before dorsal closure begins, amnioserosal cells undergo cycles of apical constriction and retraction, and tension in the amnioserosa appears to feed back on this cycling behavior, as cutting amnioserosal cells arrests or weakens neighboring cells' contraction cycles (Solon et al., 2009).

Actin and myosin regulators have been identified as players in amnioserosal movements (Jacinto et al., 2002), including a Rac GTPase that functions specifically in

apical constriction of the amnioserosal cells (Harden et al., 2002). Overexpression of a constitutively active form of Rac leads to overconstriction of the amnioserosal cells, and the cells then begin to pull away from the leading edge of the epidermis. Interestingly, in contrast to these studies, it has also been found that Rac triple mutant embryos do not have defects in amnioserosal cell contraction (Woolner et al., 2005). Thus the role of Rac signalling in amnioserosal cells is still unclear. Further studies using a myosin heavy chain (Zip) mutant reveals that amnioserosal cells that do not express this myosin II fail to apically constrict, remaining rounded (Franke et al., 2005). Rho1 and Dia also play roles in amnioserosal cell constriction, both stabilizing F-actin and activating myosin (Homem and Peifer, 2008). Dpp signaling through the Type I receptor thick veins (tkv) activates this contraction (Fernandez et al, 2007), although how it does so is not yet clear. Integrins are also required to adhere the epidermis and the amnioserosal cells during this movement (MacKrell et al., 1988; Hutson et al., 2003).

Wound healing is a process that requires cell shape changes and coordinated cell movements. Like dorsal closure, wound healing requires the spreading and fusion of epithelial sheets. Wound healing involves forces provided by a contractile, supracellular purse string (Redd et al., 2004; Martin and Parkhurst, 2004; Clark et al., 2009), but a clear, primary role for apical constriction of cells in the wound exists, at least in one system (Davidson et al., 2002). During wound healing in the animal cap of *Xenopus* embryos, F-actin accumulates in a purse string around the wound margin. Davidson et al. have performed elegant experiments to test whether the purse string or apical constriction of deep cells drives wound closure in *Xenopus* (Davidson et al., 2002). If a supracellular actin purse string mechanism provides the force, there would be at least two predictions.

First, if a square wound is generated, with sharp corners, then as the wound shrinks, purse string forces should cause the wound profile to become rounded. Second, the wound margin should be under tension as it closes. To test the first prediction, the authors made square and rectangular wounds. As the wounds healed, they maintained squared corners, and a triangular wound even closed through a Y-shaped intermediate. Second, a wound was created, allowed to heal for 15 minutes, and then two nicks were made across the purse string cable. Perhaps surprisingly, no recoil was observed, and the rate of wound closure was unaffected. If a purse string does not provide the force for closure, what does? Davidson et al. propose that contraction of the apical surfaces of cells deep in the wound provides a driving force for wound closure (Figure 1.9).

Vertebrate neural tube formation: hingepoint cells bend a sheet

Formation of the neural tube is a complex morphogenetic process that involves a diverse collection of cell movements and cell shape changes, both extrinsic and intrinsic to the neuroepithelium (reviewed in Sadler, 1998). There are two mechanisms by which the neural tube forms, known as primary and secondary neurulation. Primary neurulation occurs in the brain and future trunk region, and refers to the folding of the neuroepithelium into a tube. Secondary neurulation occurs in the posterior neural tube and refers the condensation of mesenchymal cells into a solid rod, followed by an epithelial transition into a tube (Lowery and Sive, 2004). Mechanisms of neural tube formation are of added interest because failure of the neural tube to close is a leading cause of congenital birth defects (Detrait et al., 2005; Harris and Juriloff, 2007). Of particular interest to this review is primary neurulation, in which a group of cells in the neuroepithelium, known as hingepoints cells, apically constrict, aiding in the bending of the neural plate. There are two types of hingepoint cells in the neural tube: the medial

hingepoint and the paired dorsal lateral hingepoints. The medial hingepoint is established in the ventral neural tube, and forms the neural groove (Schoenwolf and Smith, 1990) (Figure 1.2, Figure 1.10). Paired dorsal lateral hingepoints are found at the base of the neural folds (Figure 2), where the neural plate bends around the dorsal lateral hingepoints and the neural folds converge (Schoenwolf and Smith, 1990) (Figure 10).

Although the spatio-temporal development of hingepoint cells vary between model systems, hingepoint cells share a common description: these cells undergo a distinct change in cell shape in which cells become wedged, and the apical surfaces narrow. This occurs in a variety of vertebrate systems, including amphibians (Baker and Schroeder, 1967; Burnside, 1971; Schroeder, 1970), birds (Karfunkel, 1972; Schoenwolf and Franks, 1984) and mammals (Moore et al., 1987; Morriss-Kay, 1981; Shum and Copp, 1996). Patterns of bending in the neural tube have been shown to correlate with regions of apical constriction in the neuroepithelium (Bush et al., 1990; Nagele and Lee, 1987). Dense distributions of microfilaments have been observed under apical surfaces of neuroepithelial cells, leading to the long-standing hypothesis that a contractile network is responsible for hingepoint cell apical constriction (Baker and Schroeder, 1967; Schroeder, 1970; Freeman, 1972; Burnside, 1973; Schroeder, 1973; Nagel and Lee, 1980). In fact, early studies that disrupted the actin cytoskeleton by cytochalasins (Karfunkel, 1972; Morriss-Kay and Tuckett, 1985; Morriss-Kay, 1981) or by increased hydrostatic pressure (Messier and Seguin, 1978) resulted in disruption of neural tube closure. More recent work has built a molecular pathway supporting the hypothesis that a contractile network drives apical constriction. Similar to some examples of apical constriction from other systems, discussed above, key proteins that play roles in

actomyosin contraction are localized apically, including Rho, Rho-kinase (ROCK), and the motor protein myosin IIB (Hildebrand, 2005; Kinoshita et al., 2008; Nishimura and Takeichi, 2008). Importantly, the myosin II motor complex is not only localized apically, but is also active at the apical surface of the neuroepithelium, as observed by phosphorylation of the myosin II light chain (p-MLC) (Kinoshita et al., 2008; Nandadasa et al., 2009; Nishimura and Takeichi, 2008). Other cytoskeletal regulators, including Abl/Arg (Koleske, et al., 1998), Mena/Vasp (Menzies et al., 2004), and MARCKS (Zolessi and Arruti, 2001), are known to function in neural tube closure, but whether these function specifically in apical constriction is unclear (Harris and Juriloff, 2007).

Studies of the protein Shroom3 have been valuable in demonstrating a conserved vertebrate regulator of apical constriction in the neural tube. Shroom3 was first identified in mouse as a mutation that prevented the convergence of neural folds predominantly but not exclusively in the cranial region, leaving neural folds "mushroomed" away from the midline (Hildebrand and Soriano, 1999). In mice, the expression of *Shroom3* is dynamic in the neuroepithelium, and it is expressed in several other tissues including the somites, ventral body wall, heart, and gut (Hildebrand and Soriano, 1999). In *X. laevis*, *Xshroom3* RNA expression is initiated in the anterior neural plate and extends posteriorly (Haigo et al., 2003). Within the neural plate, *Xshroom3* is expressed in the superficial layer (Lee et al., 2009), where cells undergo apical constriction. Shroom3 protein expression overlaps with F-actin at both stress fibers and adherens junctions in primary neural tube cells (Hildebrand and Soriano, 1999) and localizes to the apical junctions of the neural epithelium (Hildebrand, 2005; Nishimura and Takeichi, 2008).

Shroom3 functions as an apical determinant, required for the apical accumulation of F-actin, myosin IIB, Rock1, and pMLC in the neural tube (Haigo et al., 2003; Hildebrand, 2005; Hildebrand and Soriano, 1999; Nishimura and Takeichi, 2008). In addition, Shroom3 can induce a redistribution of the microtubule regulator γ-tubulin, and is required for the assembly of apically localized parallel microtubule arrays required to drive apicobasal elongation of neural tube cells (Lee et al., 2007). However, Shroom3 is not required for apical ZO-1 localization, indicating that Shroom3 is not essential for all aspects of apicobasal cell polarity (Hildebrand, 2005; Lee et al., 2007; Nishimura and Takeichi, 2008). Shroom3 expression is sufficient to drive apical constriction in undifferentiated and transcriptionally quiescent polarized blastula cells in *X. laevis* (Haigo et al., 2003), and induces wedge-shaped cells in MDCK cell cultures (Haigo et al., 2003; Hildebrand, 2005). Interestingly, it is likely that Shroom3 expression alone does not determine the identity of hingepoint cells in the neuroepithelium as expression of Shroom3 in mouse and chick does not appear to be restricted to cells undergoing apical constriction (Hildebrand, 2005; Nishimura and Takeichi, 2008).

How does Shroom3 drive apical constriction? Shroom3 binds to and recruits ROCKs to the apical junctions (Nishimura and Takeichi, 2008). When the interaction between Shroom3 and ROCK was antagonized, pMLC failed to accumulate apically and neural tube closure was disrupted (Nishimura and Takeichi, 2008). Rhos are known activators of ROCKs (reviewed in Riento and Ridley, 2003), and thus a reasonable hypothesis is that Rho is required for Shroom3-mediated apical constriction. However, dominant negative constructs that block Rho signaling do not affect Shroom3-mediated apical constriction (Haigo et al., 2003; Hildebrand, 2005). Instead, Rap1 and possibly Ras

are required for Shroom3 dependent apical constriction (Haigo et al., 2003). These studies do not exclude a role for Rho in hingepoint apical constriction. Rho is, in fact, found apically in the neuroepithelium, and may show a slight accumulation at the hingepoints (Kinoshita et al., 2008). When Rho signaling was blocked by the addition of C3 toxin, the myosin II motor complex was not active, and the neural tube failed to close (Kinoshita et al., 2008). Additional studies will be necessary to resolve the function of Rap1 and Rho signaling during actomyosin contraction in neuroepithelial cells, and to further define the role of Rap1 in Shroom3-mediated apical constriction.

Despite evidence for a contractile actomyosin network regulating apical constriction in the neural tube, studies have shown that the requirement for F-actin during neural tube closure is not a strict one. In chick embryos treated with cytochalasin D, wedging of dorsolateral neuroepithelial cells and convergence of neural folds were blocked, but medial hingepoints were unaffected in the absence of apical microfilaments (Schoenwolf et al., 1988). Similarly, in mouse embryonic cultures, cytochalasin D treatment prevented neural tube closure at the cranial region, but the formation of medial hingepoints and dorsal lateral hingepoints continued in the spinal region, and spinal neurulation proceeded (Ybot-Gonzalez and Copp, 1999). Thus, the formation of hingepoints and bending of the neuroepithelia in cytochalasin D treated embryos suggests that apical constriction in some hingepoint cells may be actomyosin independent. Alternative mechanisms for creating hingepoints cells have been proposed, including expansion of the basal membrane through nuclear movement. Cells at the medial hingepoint progress through the cell cycle, but there is an accumulation of cells with longer cell cycles and shorter mitotic stages (Smith and Schoenwolf, 1988). Using the

observation that mitosis occurs at the apex of the neural plate, Smith and Schoenwolf suggest a model in which cells in the medial hingepoint have lengthened cell cycles, thus the nuclei are positioned basally for longer periods (Smith and Schoenwolf, 1988). This basal expansion may function to narrow the apical surface in relation to the basal surface, but it remains unclear whether this contributes to the forces that result in hingepoint formation and bending of the neuroepithelium.

Many interesting questions remain concerning both the molecular and cellular mechanisms of hingepoint formation. There is some evidence that indicates F-actin is localized basally before apical enrichment: F-actin is more concentrated at the basal sides in mouse when the cranial neural tube is in a biconvex morphology (Sadler et al., 1982), and in chick at the prospective medial hingepoint (Zolessi and Arruti, 2001). During later stages of chick neural tube formation, F-actin and pMLC show a reciprocal pattern, in that F-actin is apically localized and pMLC is predominantly basal. As neural tube formation persists, pMLC becomes apically localized (Sai and Ladher, 2008). Interestingly, myosin II regulatory light chain also becomes relocalized from the basal side to the apical side in *Drosophila* during apical constriction of the ventral furrow cells (Nikolaidou and Barrett, 2004). The function of this cytoskeletal reorganization in the neural tube is currently unclear. In *X. laevis*, myosin heavy chain B (MHC-B) is found cortically, with a concentration at the basal surface in neuroepithelial cells (Rolo et al., 2009). Knockdown of MHC-B disrupted apical F-actin accumulation and apical constriction of the neuroepithelial cells (Rolo et al., 2009). Depleting MHC-B increased deformability of the neural tissue, possibly by interfering with myosin IIB's role in cortical integrity (Rolo et al., 2009).

Adhesion, both at cell-cell junctions and at cell-matrix junctions, is likely to be important in apical constriction, and adhesion proteins and regulators have been identified as important players in apical constriction in the neural tube. Mutations in p190 RhoGAP, a mediator of integrin-dependent adhesion, result in excess basal accumulation of F-actin in the neuroepithelium, and apical constriction and neural tube closure are affected (Brouns et al., 2000). The *X. laevis* homolog of Enabled (Xena) is enriched at cell-cell junction complexes, and is required for apical F-actin accumulation, as well as for apical constriction in the neuroepithelium and cell adhesion (Roffers-Agarwal et al., 2008). Depletion of N-cadherin in the neural plate causes neural tube closure defects in *X. laevis*; however, cell adhesion is not obviously affected, possibly due to the presence of C-cadherin in the neural plate (Nandadasa et al., 2009). In neural plate cells with diminished N-cadherin, apical F-actin and phospho-myosin regulatory light chain distributions were disrupted and the apical surface areas increased, suggesting a loss of cortical tension (Nandadasa et al., 2009).

Further studies are needed to understand the pathways leading to hingepoint formation and apical constriction in the neuroepithelium, and the interplay between actomyosin contraction and cell adhesion. Cell fate is likely to have a role in determining precisely which cells in the neuroepithelium will apically constrict. Studies have shown that the secreted signal Sonic hedgehog (Shh), emanating from the notochord, and BMP, expressed in the surface ectoderm overlying the spinal neural folds, can inhibit the formation of dorsal lateral hingepoints, while the BMP antagonist Noggin induces dorsal lateral hingepoint bending (Ybot-Gonzalez et al., 2002; Ybot-Gonzalez et al., 2007). In zebrafish, the ventral expression border of *zic2a*, a transcription factor, appears to predict

the location of the dorsal lateral hingepoints (Nyholm et al., 2009). An important area of future research will be to determine what factors cause hingepoint cells to apically constrict or prevent the apical constriction of neighboring cells.

CONCLUSIONS

Cell and developmental biologists have come a long way toward building an understanding of apical constriction, from the observations and hypotheses of Rhumbler in 1902, through physical and chemical perturbations, to building genetic pathways and dissecting protein functions, and into an age in which such findings can be integrated with biochemical mechanism and an understanding of force production. This kind of integration is likely to be important to gain a real understanding of the mechanisms by which development regulates the morphogenetic forces that shape animals. The connections established between patterning and morphogenesis are valuable steps toward defining the general rules by which forces are spatially regulated by developmental programs.

What can we conclude so far about common themes and variations? One commonly-demonstrated mechanism for the cell shape change of apical constriction is the localization and activation of myosin on an F-actin meshwork on the apical sides of cells. Mechanisms of spatial regulation of this common mechanism appear to vary widely between organisms and between tissues within an organism. A highly contractile actomyosin network can be localized to the apical side of a cell based on diverse sources of apicobasal polarity information, such as the apically-polarized secretion of Fog protein in *Drosophila*, apicobasal PAR protein localization in *C. elegans*, or apical Shroom

localization in *Xenopus*, mouse and chick. Some proteins identified to date seem unlinkely to play conserved roles across the metazoa, as large groups of animals may lack key proteins. For example, Shroom is not yet known to exist outside of deuterostomes and arthropods (Dietz et al., 2006), and Fog appears to be a *Drosophila*-specific protein (Costa et al., 1994). Which cells will undergo apical constriction can also be determined by diverse sources of cell fate information, often involving transcriptional regulation, for example by GATA factor proteins in *C. elegans* gastrulation, bHLH and zinc finger proteins in *Drosophila* gastrulation, and a *Drosophila* bHLH/PAS protein in trachea formation. These findings suggest that common cytoskeletal mechanisms driving apical constriction are regulated by a variety of patterning mechanisms (Figure 2).

Apical constriction can play central roles in diverse morphogenetic movements, including the internalization of small numbers of cells, the bending a tissue into a folded sheet, and the initiation of tube formation. One common theme is that apical constriction is used frequently in gastrulation. Of course, other classes of morphogenetic movements are often used in gastrulation as well. Given the diverse regulators identified to date, it will be difficult to estimate the extent to which gastrulation in systems like *C. elegans* and *Drosophila* are conserved modifications of an ancestral mechanism, as opposed to independent co-option of apical constriction mechanisms, until mechanisms are compared in relatives of these organisms.

Contraction of an apical microfilament network is not the only way apical constriction can take place. A shrinking of the apical side of a cell may also be driven by basolateral lengthening or expansion, movement of apical surface to lateral domains, or by extracellular forces, as discussed. The forces that can drive apical constriction do so in

a mechanical context that can result in shrinking of only apical surfaces, rather than causing cell columnarization, for example, and such mechanical contexts have been explored only rarely (Davidson et al., 1999; Ma et al., 2009, for example). Thus, many questions remain. How much do these other processes act as primary drivers of apical constriction, and how much do they participate alongside constriction of an apical actomyosin meshwork? Forces from multiple cells can also contribute to a morphogenetic process, such as dorsal closure in flies. How are multiple forces coordinated to drive morphogenesis? What determines the degree of redundancy used to drive a morphogenetic event? Redundancy is a theme developmental biologists are increasingly able to address with new tools. Despite the apparent simplicity of apical constriction, redundant mechanisms are often involved. New computer models, building on the brass bar and rubber band models of Lewis, may be able to incorporate redundant mechanisms that may drive morphogenetic movements, and this may become increasingly important for testing the feasibility of hypotheses and for suggesting key experiments in the future. Likewise, more sophisticated experimental analyses of the cellular and multicellular mechanics will lead to better and more accurate models.

FIGURES

Figure 1.1

Rhumbler's 1902 drawings of cell shape changes driving morphogenesis. Top: A sea urchin embryo undergoing primary invagination. The vegetal-most part of the embryo bends inward (arrowhead). Bottom: "Theoretical gastrulation scheme, to show that invagination (b) of a cell plate (a) necessarily must take place if each cell changes from form a1 (due to higher pressure on the pigmented side) to the form b1. The invagination effect is significant even though the change in cell form from a1 to b1 is very small" (translation of figure legend in Rhumbler, 1902). We have inverted some parts of this figure to match the orientation of tissue bending between drawings.

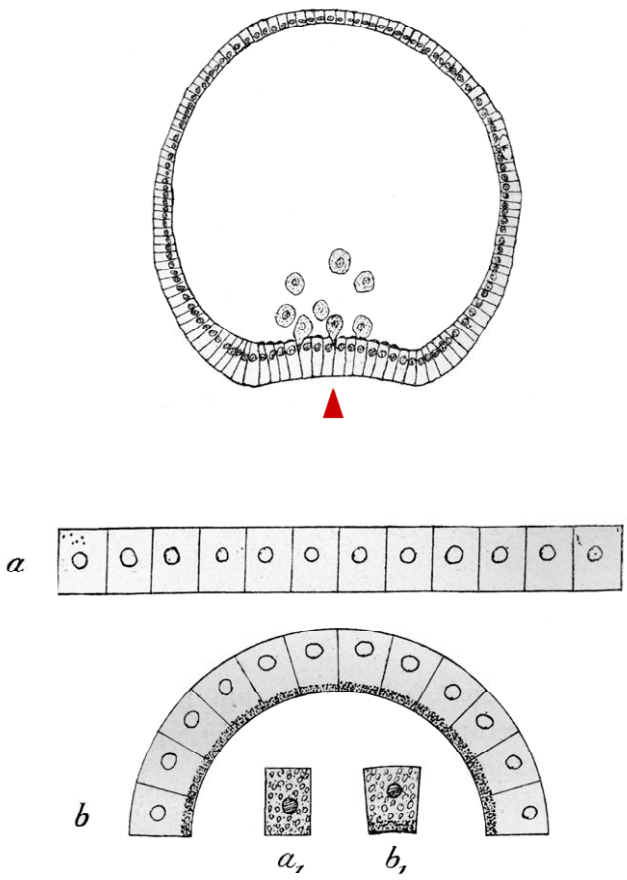
a
b
a,
b,

Figure 1.2

Scanning electron micrographs of apically constricting cells in diverse systems. A) Sea urchin vegetal plate (Kimberly and Hardin, 1998), B) *X. laevis* midsagittal section at early gastrula showing bottle cells (BC) and involuted mesodermal cell stream (MCS) (Keller, 1981) C) *Drosophila* ventral furrow formation (Sweeton et al., 1991), D) Chick neuroepithelial medial and dorsal lateral hinge points. Scanning electron micrographs of transverse slices through the medial (left) and dorsal lateral (right) hinge points at the future hindbrain level; asterisk, notochord; w, s, wedge- and spindle-shaped cells, respectively (Schoenwolf and Smith 1990). Arrowheads mark bends in epithelia at proposed sites of apical constriction.

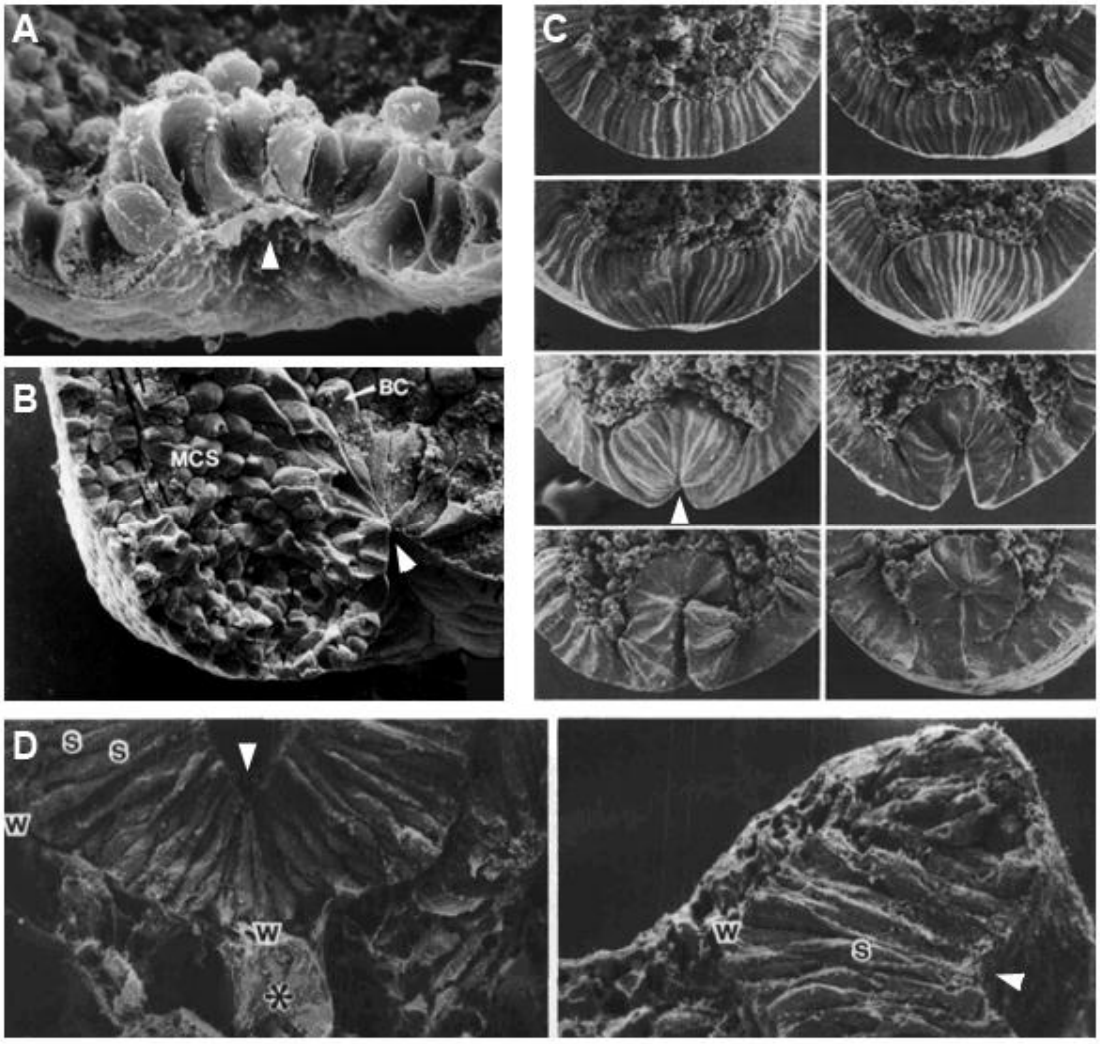
A
B
BC
MCS
C
D
S
S
W
W
W
S

Figure 1.3

Some of the known genetic pathways by which cell fate and cell polarity regulate apical constriction, in three selected systems (Lee et al., 2004; Lecuit, and Lenne, 2007; Lee et al., 2007; Anderson et al., 2008; Chung and Andrew, 2008, and references in text). For simplicity, some other mechanisms for apical constriction are not diagrammed, and important links between contractile actomyosin networks and cell-cell adhesion proteins are omitted here.

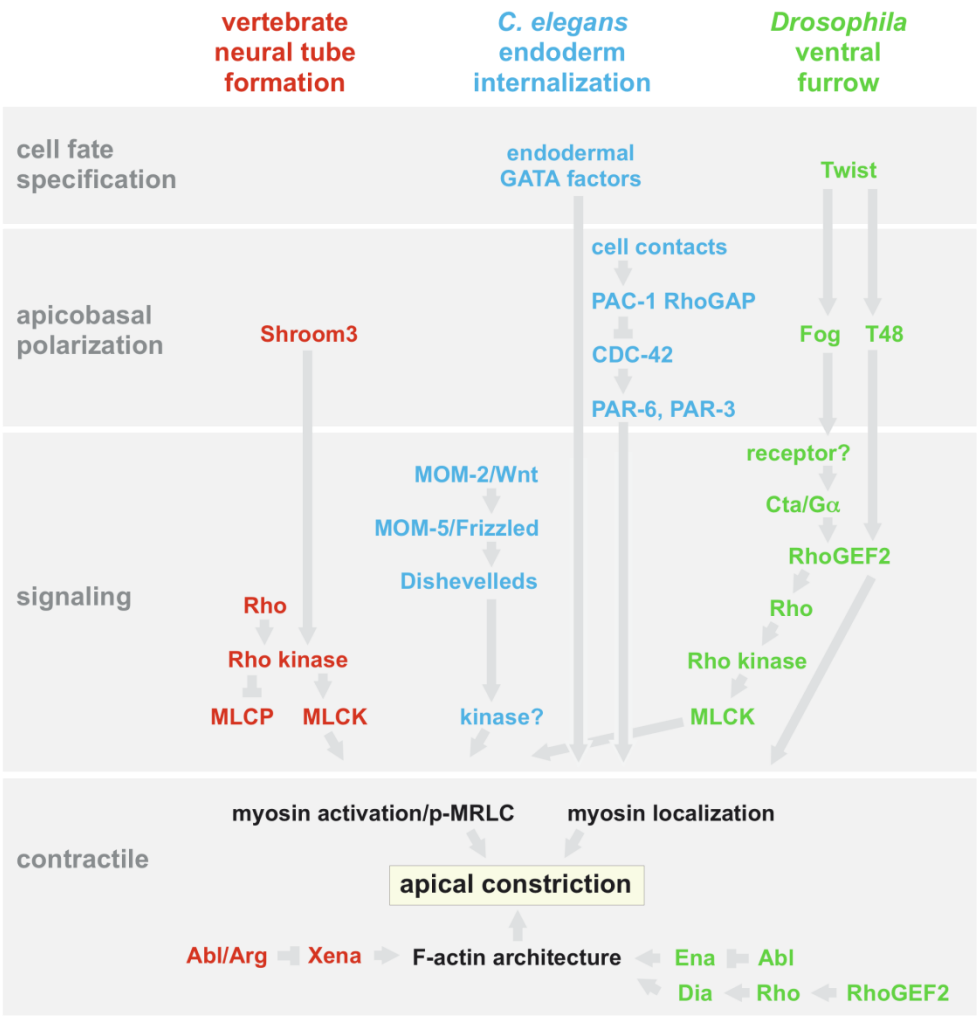
vertebrate neural tube formation
C. elegans endoderm internalization
Drosophila ventral furrow
cell fate specification
endodermal GATA factors
Twist
apicobasal polarization
cell contacts
PAC-1 RhoGAP
CDC-42
PAR-6, PAR-3
Shroom3
Fog
T48
signaling
receptor?
MOM-2/Wnt
Cta/Gα
MOM-5/Frizzled
RhoGEF2
Dishevelleds
Rho
Rho
Rho kinase
Rho kinase
MLCP
MLCK
kinase?
MLCK
contractile
myosin activation/p-MRLC
myosin localization
apical constriction
Abl/Arg
Xena
F-actin architecture
Ena
Abl
Dia
Rho
RhoGEF2

Figure 1.4

Schematic diagrams of bottle cell formation. All images approximate midsagittal views. A) Prior to gastrulation, the prospective anterior mesoderm (darker shading) and posterior mesoderm (lighter shading) comprise the deep marginal zone. B) The bottle cells have undergone apical constriction. Arrows indicate movements hypothesized to result. C) This causes reorientation of the vegetal edge of the marginal zone (anterior mesoderm) such that it is now leading the movement into the blastocoel (Hardin and Keller, 1988).

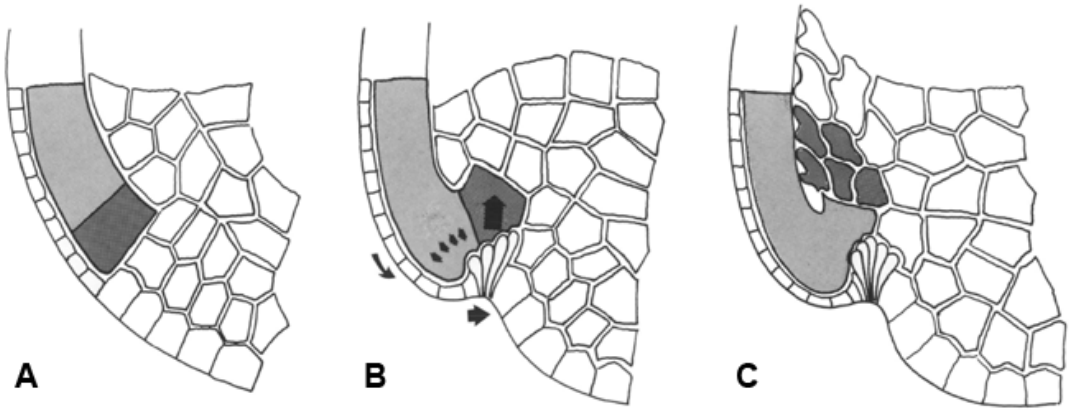

Figure 1.5

C. elegans gastrulation. A) Illustrations of embryos just before (top) and during (bottom) endodermal internalization. Green, endodermal progenitors. Two neighboring cells are marked in purple. Renderings by J. Iwasa based on confocal sections of phalloidin-stained embryos (Lee et al., 2006). B) Diagram showing where apical constriction occurs (arrowheads). C) Myosin is activated in the apical cortex of the internalizing cells. Phospho-regulatory myosin light chain staining is in green (Lee et al., 2006).

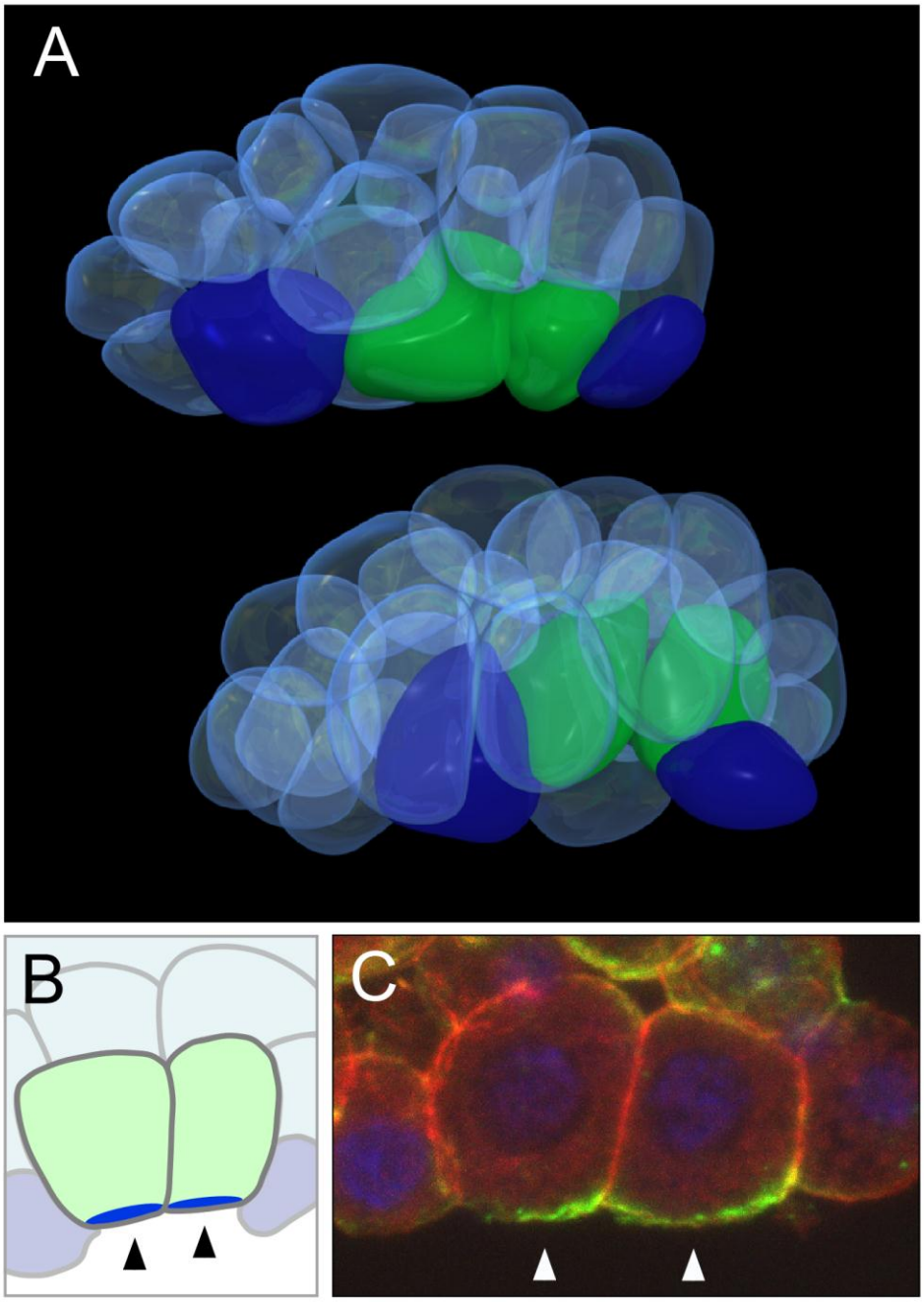
A
B
C

Figure 1.6

Forces driving *Drosophila* ventral furrow invagination. Tracings of transversely fractured scanning electron micrographs. Cells that apically constrict are colored yellow. Adjacent cells in the ventral plate that develop flattened apical surfaces but do not constrict apically are colored red-orange. Small arrows outside or within cells represent the presumed vectors of forces within these cells as a result of apical constriction or cell elongation or shortening. Larger arrows indicate cumulative forces predicted from the combined forces of individual cells (Costa et al., 1993).

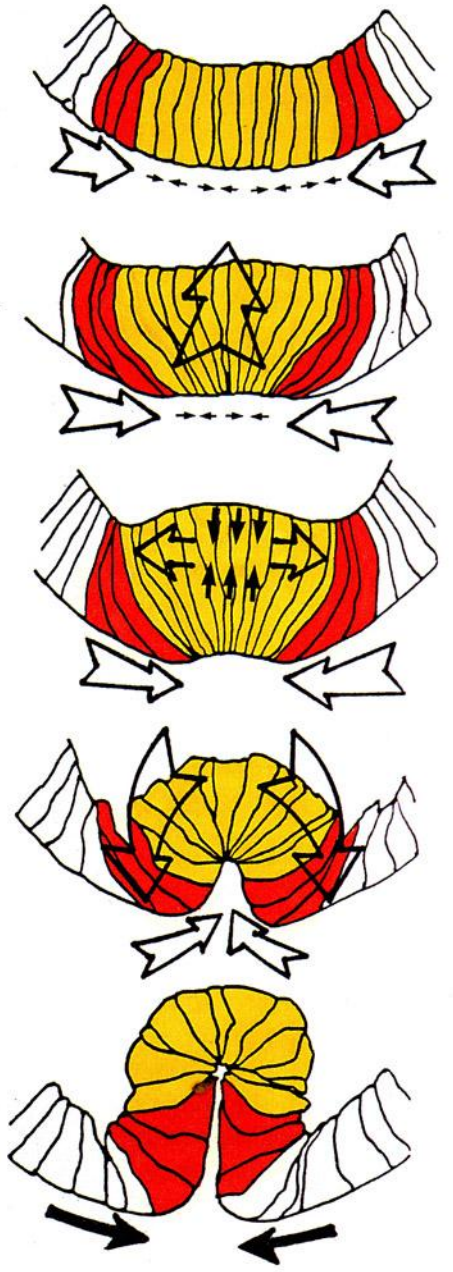

Figure 1.7

Apicobasal shortening of cells within the morphogenetic furrow. Schematic of a cross section through the eye imaginal disc. Columnar cells of the eye imaginal disc epithelium are apically constricted and shorter within the morphogenetic furrow (MF, blue cells). Dark blue lines indicate zonula adherens. A layer of squamous cells, the peripodial membrane (PM), overlies the columnar cells (Schlichting and Dahmann, 2008).

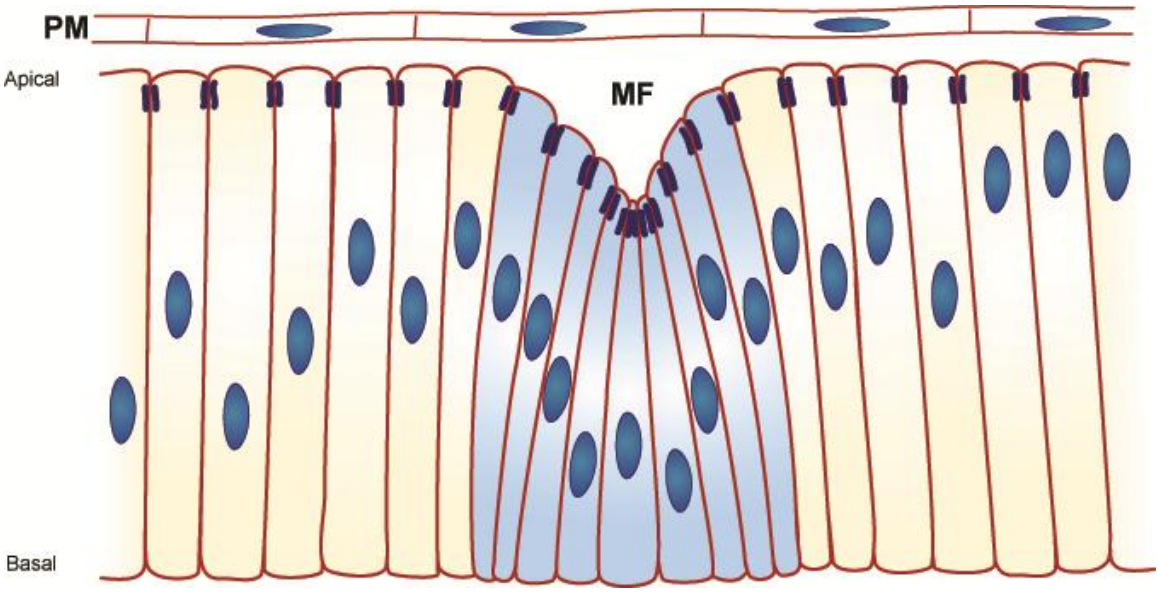

Figure 1.8

Schematic of cell shape changes during tracheal invagination. The dark line delineates the apical surfaces of the cells. Before invagination (left), cells form a flat epithelium. At the onset of invagination (middle), a small group of cells have apically constricted (red arrowhead). The invagination lengthens into a tube that turns dorsally (right) (after Brodu and Casanova, 2006).

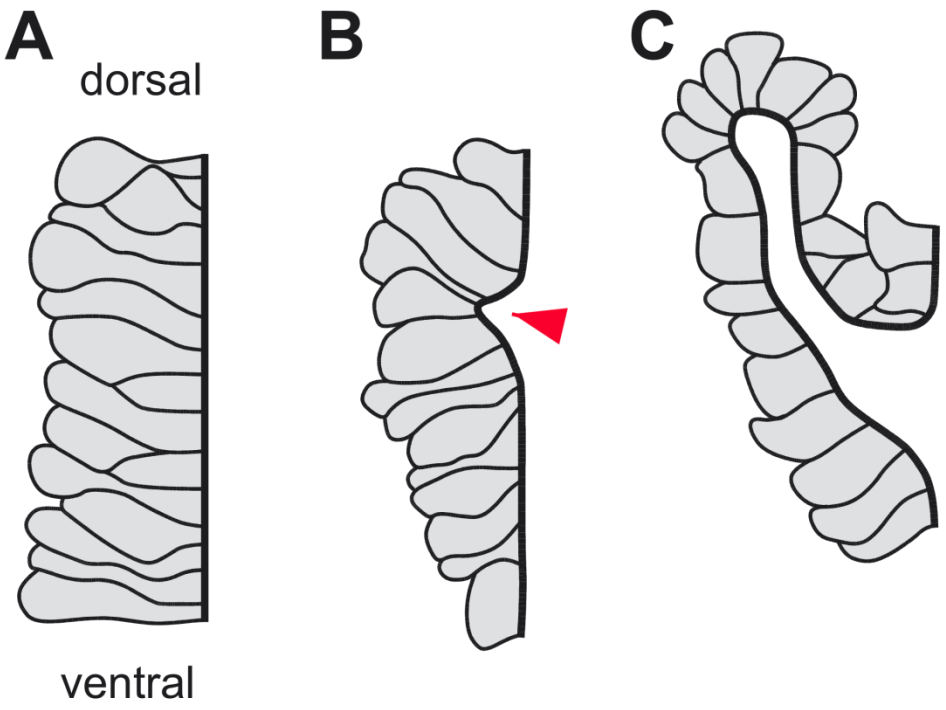
A
dorsal
ventral
B
C

Figure 1.9

Apical constriction of deep cells during epithelial wound healing. Schematic of embryonic wound healing in the *Xenopus laevis* animal cap ectoderm. An excisional wound was made that removed only the outer cell layer of the two cell-layered animal cap ectoderm. Apical constriction drives reduction in wound size (Davidson et al., 2002)

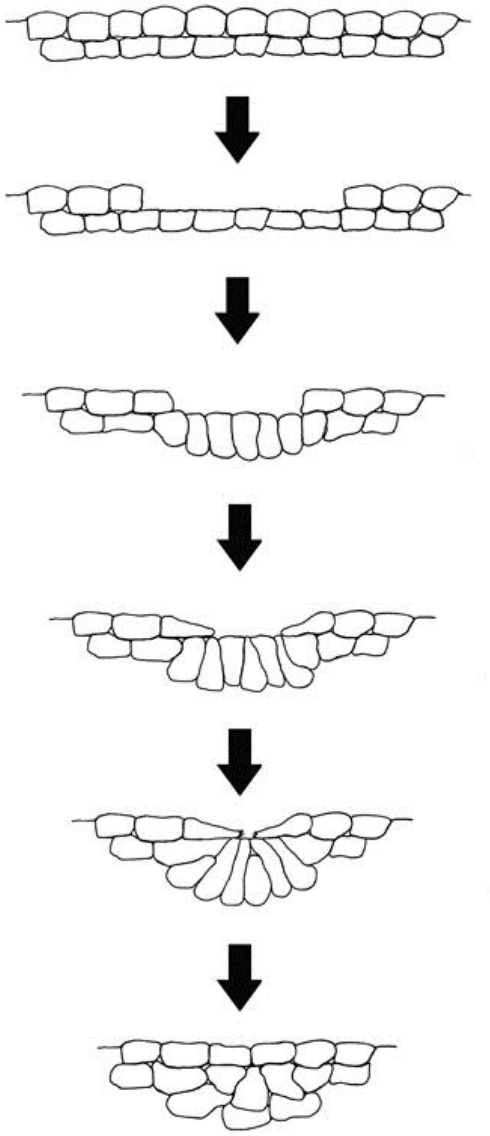

Figure 1.10

Medial and dorsal lateral hingepoint cells in vertebrate neurulation. Schematic representation of a transverse section through the future hindbrain level of a chick embryo, illustrating the characteristics of neurepithelial cells in the medial hingepoints (asterisk), dorsolateral hingepoints (double asterisks) and lateral neural plate between the hinge points; n, notochord; se, surface ectoderm (Schoenwolf and Smith 1990).

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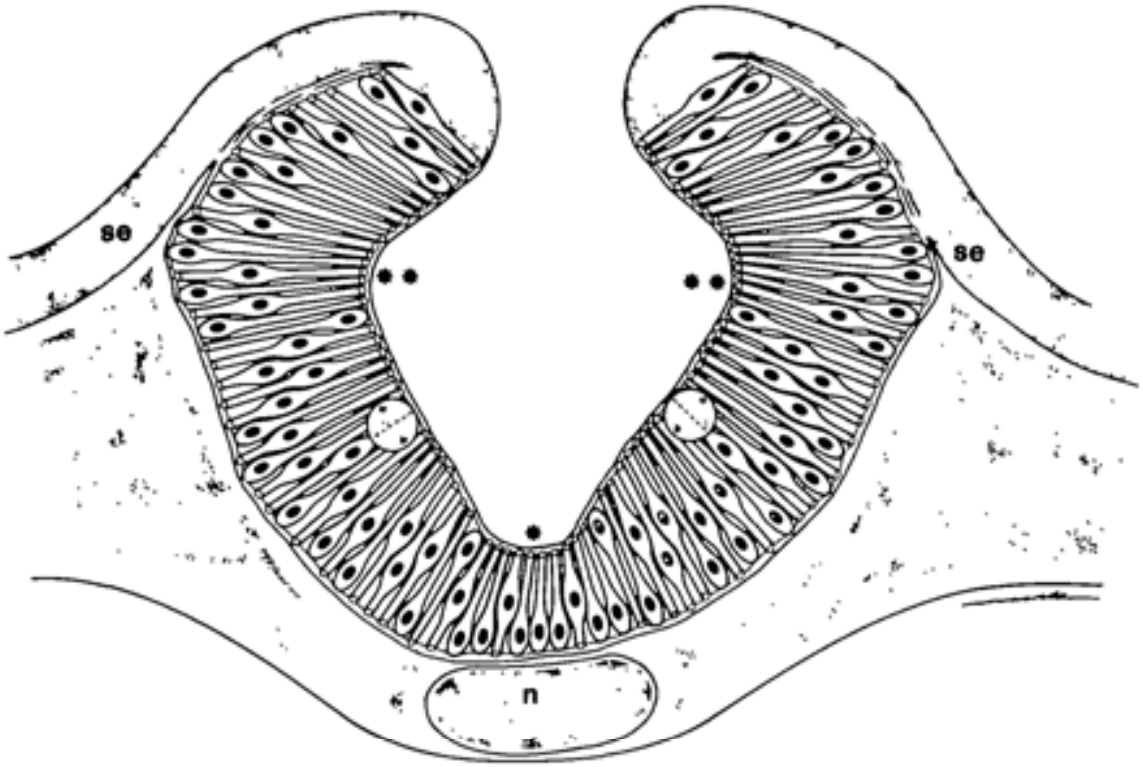

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CHAPTER 2

Overcoming Redundancy: an RNAi Enhancer Screen for Morphogenesis Genes in *C. elegans*

This chapter is adapted from a manuscript accepted to the journal Genetics *(*Sawyer et al., 2010*). Noor White and Corbin Jones provided Figures 2.10, 2.14, and 2.15 and supportive text and Natalia Starostina and Edward Kipreos provided Figure 2.16 and supportive text. Two fantastic research undergraduates, Trudy Li and Stephanie Glass, helped with the feeding RNAi experiments summarized in figures 2.3, 2.4, and 2.7. I have performed the experiments for the remainder of the chapter and, together with Corbin Jones, Edward Kipreos, and Bob Goldstein, wrote the manuscript.*

ABSTRACT

Morphogenesis is an important component of animal development, yet genetic redundancy has been proposed to be a common feature of morphogenetic processes, thus posing a challenge to the genetic dissection of morphogenesis mechanisms. Here, we present a screen designed to uncover redundant and partially redundant genes that function in an example of morphogenesis, gastrulation in *Caenorhabditis elegans*. We performed an RNAi enhancer screen in a gastrulation-sensitized double-mutant background, targeting genes likely to be expressed in gastrulating cells or their neighbors.

Secondary screening was used to identify genes with detectable effects on gastrulation in both sensitized and non-sensitized backgrounds. By this method, we identified 16 new genes whose function is required for normal gastrulation in a non-sensitized background. We observed that for most of these genes, their closest known homologs were multiple other *C. elegans* genes, suggesting that some of these genes may have derived from rounds of relatively recent gene duplication events. We predict that such genes are more likely than single copy genes to comprise redundant or partially redundant gene families. We explored this prediction for one of the new genes. Our results confirmed that this gene and five close relatives do indeed function partially redundantly with each other in gastrulation. Our results implicate new genes in *C. elegans* gastrulation, and show that an RNAi-based enhancer screen can be used as an efficient means to identify important but redundant or partially redundant developmental genes.

INTRODUCTION

Morphogenesis involves cell and tissue movements, including the movements of gastrulation and neurulation in animal embryos. Identifying the genes that control morphogenesis in animal systems has been a long-standing challenge (Wieschaus, 1997). Genes involved in morphogenesis may evade genetic screens for at least two reasons: first, some genes controlling morphogenesis encode widely pleiotropic proteins such as actin and myosin (Kiehart et al., 1990). These genes may be missed in screens for morphogenesis genes because loss of function can result in arrested development before morphogenesis begins. Other genes may have functions that are too subtle to be identified in forward screens, for example genes that function redundantly or partially redundantly.

Redundancy among mechanisms that underlie morphogenesis has been called a "well-recognized aspect of development" (Newman and Comper, 1990). In his Nobel Lecture, Eric Wieschaus concluded that classic *Drosophila* screens failed to identify many morphogenesis genes, and proposed as a result that the control of cell form that underlies morphogenesis may be unusually susceptible to genetic redundancy (Wieschaus, 1997). Redundancy is a challenge that biologists face increasingly, as large proportions of genes in diverse systems have been found to perform important functions as members of redundant gene groups, and as a result, are often missed in genetic screens (Johnsen and Baillie, 1997; Rutherford, 2000; Gu et al., 2003; Felix and Wagner, 2008). We recognize that two distinct forms of genetic redundancy exist: homologous redundancy, in which homologous proteins can substitute for each other, and non-

homologous redundancy, in which proteins that do not resemble each other can substitute for each other, for example by affecting distinct, contributing cellular mechanisms (Jorgensen and Mango, 2002; Gu, 2003).

Despite this challenge, some key genes that function in morphogenesis have been identified by standard forward screens and by a variety of elegant modifications of such screens (Metzger and Krasnow, 1999; Beitel and Krasnow, 2000; Starz-Gaiano and Montell, 2004; Zohn et al., 2005; Maybeck and Roper, 2009; Ellertsdóttir et al., 2010; Rochlin et al., 2010; Szabo-Rogers et al., 2010, for example). *Caenorhabditis elegans* is a valuable model system for contributing to this effort, because genetics and RNA interference (RNAi) allow one to simultaneously disrupt the functions of multiple genes in modifier screens (Labbé et al., 2006; O'Rourke et al., 2007; Dorfman et al., 2009). Genetic modifier screens have identified genes with redundant roles in *C. elegans* vulval and pharyngeal morphogenesis (Fay and Yochem, 2007). To our knowledge, RNAi modifier screens have not yet been used to find genes controlling morphogenesis, or to specifically seek redundant and partially redundant groups of genes. The ability to observe directly the individual cells participating in morphogenesis in transparent *C. elegans* embryos *in vivo* (Chisholm and Hardin, 2005; Nance et al., 2005) makes it possible to detect even subtle defects. Detecting subtle defects may be important for identifying partially redundant genes.

Gastrulation is a key morphogenetic event, a cellular reorganization that occurs in diverse metazoans. Gastrulation involves the internalization of cells that give rise to mesoderm, endoderm and germline, leaving these cells enclosed by ectoderm. In *C. elegans*, gastrulation begins with the internalization of two endodermal precursor cells,

Ea and Ep, from the ventral face of the embryo. These two cells are the first cells of the embryo to introduce in their cell cycles a gap phase, during which they internalize (Edgar and McGhee, 1988). Six neighboring cells, including the germline precursor (P_4), three of the four granddaughters of the MS founder cell, and two great-great-granddaughters of the AB founder cell move into the space that the internalizing E cells leave behind, completing envelopment of the Ea and Ep cells (Lee and Goldstein, 2003). Sixty-four other cells internalize after the endoderm precursors, leading to roughly half of the embryonic cells ending up in the interior of the embryo (Sulston et al., 1983; Nance and Priess, 2002; Harrell and Goldstein, in press).

C. elegans gastrulation requires properly specified cell fates and involves cell polarization, control of motor activity, regulation of adhesion, and mechanistic links from cell fate specification to cell movements. One genetic requirement for *C. elegans* gastrulation is a class of genes controlling cell fate specification. The endodermal GATA factor END-3 and genes regulating its expression in the endodermal lineage are required for timely gastrulation (Bowerman et al., 1992; Thorpe et al., 1997; Maduro et al., 2005; Lee et al., 2006). Gastrulation in *C. elegans* also depends on genes encoding PAR polarity proteins: loss of PAR-3 in somatic cells results in Ea and Ep failing to internalize on schedule (Nance and Priess, 2002). These cells normally accumulate a non-muscle myosin heavy chain protein in their apical cortex, and this accumulation requires apical PAR proteins, which localize to contact-free surfaces via a RhoGAP-mediated exclusion of PAR-6 from other surfaces (Nance and Priess, 2002; Nance et al., 2003; Anderson et al., 2008). Basolaterally-localized adhesion proteins also function in apical myosin localization (Grana et al., 2010). A WD repeat protein, GAD-1, (gastrulation defective),

is required to delay entry into mitosis during a period of apical myosin accumulation, and is required for cell internalization (Knight and Wood, 1998; Nance and Priess, 2002; Lee et al., 2006). Gastrulation additionally depends on a Wnt-Frizzled signaling pathway that activates the apical myosin in Ea and Ep (Lee et al., 2006). These results have led to a model in which myosin enriches at the apical, contact-free cell cortex of endodermal precursors, and activation of myosin results in an actomyosin-dependent constriction of the apical surface of these cells, driving movement of the cells to the embryo interior (see Rohrschneider and Nance, 2009; Sawyer et al., 2010 for review). Consistent with this model, F-actin and actin regulators also function in gastrulation (Lee and Goldstein, 2002; Severson et al., 2002; Karabinos et al., 2003; Roh-Johnson and Goldstein, 2009). Several of the genes identified to date are thought to contribute partially redundantly, as strong loss of function of genes including *end-3*, *par-3*, *par-6*, and genes of the Wnt pathway results in only a delay of E cell internalization (Nance et al., 2003; Lee et al., 2006).

We hypothesized that many genes that play direct or indirect roles in normal gastrulation remain to be identified. A screen aimed specifically at identifying *C. elegans* gastrulation genes has not been reported previously. Here, we report a novel screening strategy for identifying genes with roles in *C. elegans* gastrulation. We have constructed a double mutant worm strain to serve as a sensitized background for an enhancer screen. We found that feeding these worms bacterially-produced double-stranded RNAs (dsRNAs) targeting genes involved in gastrulation succeeded in producing synthetic lethality. We exploited this sensitized background together with two published microarray analyses (Robertson et al., 2004; Baugh et al., 2005) to screen for enhancers

of the sensitized background among genes likely to be expressed in gastrulating cells and/or their neighbors before or near the time that gastrulation occurs. In secondary screens, we determined which of the genes we identified as enhancers were required for gastrulation in a non-sensitized background. This approach identified 15 new genes that function in *C. elegans* gastrulation. We show that most of these would not have been found by a traditional RNAi feeding screen. Our screen was especially effective at identifying genes whose closest relatives were multiple other *C. elegans* genes, suggesting that these genes are members of a gene family that derived from relatively recent gene duplication events. Because of their similarity and common origin, we predict that these genes are more likely to function redundantly or partially redundantly than single copy genes. We tested this hypothesis for one such family and showed that genes in this family do indeed comprise a redundant gene set required for normal gastrulation. Our results identify a set of genes that will be valuable for further study. Moreover, they suggest that *C. elegans* modifier screens using RNAi in a sensitized background can effectively identify genetic requirements for morphogenesis, including redundant gene families that are traditionally difficult to identify genetically.

MATERIALS AND METHODS

Strains and worm maintenance

Nematodes were cultured and handled as described (Brenner 1974). Experiments were performed using the following strains: wild-type N2 (Bristol), JJ1317 *zuIs3* [*end-1*::GFP], EU452 *mom-5(zu193)unc-13(e1091)/hT2I; +/hT2[bli-4(e937)let-?(h661)]III*, MT4434 *ced-5(n1812)*, MT4417 *ced-5(n1812);dpy-20(e1282)*, RB1331 *end-3(ok1448)*, GR1373 *eri-1(mg366)*, VC271 *end-1(ok558)*, RB2454 *apy-1(ok3393)*, RB2550 *ugt-23(ok3541)*, GH403 *glo-3(kx94)*, GH383 *glo-3(zu446)*, FX03627 *b0222.9(tm3627)*, FX00278 *tbx-11(tm0278)*, FX02295 *sdz-19(tm2295)*, FX01239 *sdz-31(tm1239)*, FX01226 *vet-6(tm1226)*, FX01378 *sdz-22(tm1378)*, FX01169 *sdz-28(tm1169)*, FX04187 *c10a4.5(tm4187)*, ET099 *ekEx19* [*Pcul-2*::CUL-2::FLAG::*cul-2* 3'UTR; pRF4], LP77 was constructed by by crossing *end-3(ok1448)* males with *ced-5(n1812)* hermaphrodites. *end-3(ok1448)* is a large deletion of approximately 700 bp (WormBase Release WS215 at www.wormbase.org). All strains were maintained at 20°C.

RNAi screening and quantification of embryonic lethality

RNAi by feeding was performed at 20°C according to a standard protocol, starting with L4 larvae moved every 12 hours to fresh RNAi plates (Timmins and Fire 1998; Kamath et al., 2001). Feeding strains were obtained from a dsRNA feeding library from MRC Geneservice (Kamath and Ahringer, 2003). F1 embryos and larvae were counted at least 24 hours later. Plates from a 12 hour period were counted only if lethality for a positive control, *par-6* RNAi, was above 80% for all genetic backgrounds involved. A negative control, *gfp* RNAi, was used to determine the baseline worm strain lethality fraction (W). Worm strain lethality was accounted for to find a worm strain adjusted

lethality (L), by the equation L= (1-W)*R, where R is the raw lethality resulting from a given dsRNA fed to that worm strain. Enhancement of lethality was calculated as the difference between the adjusted lethalities (for example, L for N2 subtracted from L for *ced-5;end-3*). Comparisons between worm strains were only done between corresponding 12 hour plates within the same experiment. For statistical analysis, experimental pairs were repeated in triplicate. A two-tailed Student's T-test with two-sample unequal variance (heteroscedastic) could then be assessed between the enhancement of lethality for a given bacterial strain to the enhancement of lethality of the negative control vector, L4440 expressing dsGFP.

Templates for *in vitro* transcription were generated by a two-step PCR from wild-type genomic DNA. Primers for the first step included 20 bases matching the target sequence and 15 bases of the T7 promoter sequence. The resulting PCR product was purified using a PCR purification kit (Qiagen) according to the manufacturer's recommendations. This product was used as a template for a second PCR using primers containing the full-length T7 promoter sequence. One to two micrograms of the product were then gel-purified and used as a template in an *in vitro* transcription reaction using the T7 RiboMAX™ Express RNAi System (Promega) according to the manufacturer's recommendations. The integrity of the dsRNA was assessed by gel electrophoresis, and the concentration was determined by spectrophotometry. dsRNA was injected at a concentration of 100 ng/ml into hermaphrodites using a Narishige injection apparatus, a Parker Instruments Picospritzer II and a Nikon Eclipse TE300 microscope. dsRNA was stored in 2 volumes of 100% ethanol at either -20°C or -80°C.

Microscopy and DIC imaging

For live imaging, *C. elegans* embryos were mounted on poly-L-lysine coated coverslips, supported by a 2-3% agarose pad. 4-D differential interference contrast (DIC) microscopy was carried out using a Diagnostic Instruments SPOT2 camera mounted on a Nikon Eclipse 800 microscope. Images were acquired at 1-2 µm optical sections every 1 or 1.5 min during embryogenesis and analyzed with Metamorph v.6.3r5 (Molecular Devices). Imaging was performed at 20°C–23°C for all strains. In experiments where endoderm differentiation was scored, embryos or partial embryos were examined the next day for the presence of birefringent rhabditin granules under polarized light (Babu, 1974; Laufer et al., 1980).

Sequence alignment and phylogenetic tree construction

Amino acid sequences for the genes identified in this screen and *C. elegans vhl-1*, *zif-1*, *zer-1*, *zyg-11*, along with human and mouse zyg11 homologs and *C. briggsae CBG07183*, *CBG24348*, and *zyg-11*, were aligned using CLUSTALW and MUSCLE (Chenna et al., 2003; Edgar 2004). Both algorithms produced generally poor alignments among all sequences. However, clear regions of conservation were identified among these sequences. The alignments were trimmed to these conserved sequences and the *C. briggsae* sequences were excluded. To be included in the conserved sequence alignment, we required that at least 2/3 of taxa have an aligned base. We used ProTest to determine the best model for amino acid evolution, which was JTT+ Γ (Abascal et al., 2005). We then constructed both maximum likelihood and maximum parsimony trees for the complete sequences and the trimmed conserved sequences (Guindon and Gascuel, 2003; Kumar et al., 2008). 1000 and 500 bootstraps were performed for each algorithm

respectively. Generally, the trees were congruent regardless of algorithm or sequence used. The bootstrap support, however, was best with the trimmed conserved sequence.

Comparative BLAST+ analysis

We wrote a computer program (available from the authors) to automate BLAST+ (Camacho et al., 2009) of a gene set versus the entire *C. elegans* genome, nr, or any BLAST database. BLAST+ result files were then analyzed to determine the number of unique genes in the genome hit by a particular gene. For non-*C. elegans* analyses, all nematode sequences were filtered out. Results were then analyzed using JMP (v.8, SAS, Cary NC).

Comparative sequence analysis

We compared the newly identified gene set to the Conserved Domains Database (CCD; Marchler-Bauer et al., 2009) and filtered our trimmed alignment by similarity. No one residue was conserved across all data, but several potential motifs became apparent between 50-90% stringency.

Immunostaining and confocal microscopy

F58D2.1 polyclonal antibodies were generated from rabbits expressing the 100aa polypeptide from amino acids 198-297 RFIDCSRTMMSVELLEYLLKTHRNLQGVIATMTKSDSDIYDDARALNVATFDST VRALTYFLKANKVFENGHTITKIDDFIAADSSRILNIRPCMEIIIK (Strategic Diagnostics). 80mL rabbit antisera was affinity purified to an endpoint titer of 0.72 ng/ml. Embryos were immunostained for F58D2.1 (1:1000) as described (Tenlen et al., 2008) and imaged using a Zeiss LSM510 confocal microscope with LSM software. Images were further processed with Metamorph software.

Interaction experiments

Full-length cDNA clones of *zyg-11*, *gadr-6*/*F47G4.2*, and *gadr-5*/*Y71A12B.17* were cloned into pCMV-Tag2 vector (Stratagene) to produce FLAG-fusion constructs; *cul-2*-Myc was cloned into pEGFP-N1 vector (Invitrogen), from which the GFP sequence was removed; and the HA-ELC-1/pEGFP-N1 construct was previously described (Starostina et al., 2007). Immunoprecipitation experiments from transient transfection of HEK293T cells were performed as described (Starostina et al., 2007) using anti-FLAG (M2, Sigma) antibody for the immunoprecipitation; and anti-FLAG (M2), anti-HA.11 (Covance), and anti-CUL-2 (Feng et al., 1999) for western blots. Affinity purification coupled to LC-MS/MS to identify CUL-2::FLAG-associated proteins utilized strains ET099 (expressing *Pcul-2*::CUL-2::FLAG) and N2, and was performed as previously described (Starostina et al., 2007).

RESULTS

Identifying *end-3(ok1448)* as a sensitized background

To begin to identify a sensitized background for a gastrulation screen, we sought a mutant with a subtle gastrulation defect, which might be enhanced by feeding a dsRNA targeting another gene with a role in gastrulation (Figures 2.1 and 2.11). Loss of function of either a cell fate regulator *end-3* (endodermal GATA factor) or a member of the Wnt signaling pathway *mom-5* (Frizzled) can result in a subtle gastrulation defect in which the two Ea and Ep cells delay internalization, however one cell cycle later, their daughter cells internalize as four E cells (the E4 stage) (Maduro et al., 2005; Lee et al., 2006). We quantified these subtle gastrulation defects in an allele with a large deletion in *end-3*, *end-3(ok1448)*. In 95% of these embryos, Ea and Ep divided on the surface and became internalized one cell cycle later, as four cells (Figure 2.1). We observed similar results in the strong *mom-5(zu193)* allele, with cells internalizing late at the E4 stage in 72% of embryos (Rocheleau et al., 1997) (Figure 2.1). Injection of *mom-5* dsRNA into wild-type worms nearly phenocopied the *mom-5(zu193)* allele, with cells internalizing late at the E4 stage in 61% of embryos (Figure 2.1). These results confirmed that the gastrulation defects in these backgrounds are subtle, but highly penetrant.

We discovered that targeting *mom-5* and *end-3* together by injecting *mom-5* dsRNA into *end-3(ok1448)* worms resulted in a stronger and more penetrant defect than either single treatment: in all embryos, neither Ea/Ep nor their daughter cells internalized (Figure 2.1). This strongly synergistic effect suggests that these genes contribute to

gastrulation redundantly. The result also suggested that either of these genes might be exploited as a basis for a sensitized background to screen, ideally in a viable mutant background, for enhancement of embryonic lethality, a readily scorable phenotype. *end-3* loss of function mutants generally produce viable embryos (Maduro et al., 2005), with only 6% embryonic lethality in *end-3(ok1448)* (Figure 2.2). Loss of function mutants of *mom-5* resulted in embryonic lethality (Rocheleau et al., 1997), but feeding *mom-5* dsRNA to wild-type animals produced a much weaker defect, with only 4% of embryos failing to hatch (Figure 2.12), suggesting that RNAi by feeding for *mom-5* might be a means to generate partial loss of function. We fed *mom-5* dsRNA to *end-3(ok1448)* worms and found that 24% of embryos failed to hatch, a mild but readily detectable and significant synergistic effect (P=0.027, Student's t-test). This result suggested that by feeding dsRNAs to *end-3(ok1448)* and wild-type animals in parallel, followed by quantification of embryonic lethality, an RNAi feeding screen could be carried out.

Developing a doubly sensitized background

We next determined if other mutants can produce enhanced gastrulation defects and possibly be used to generate a more sensitized background. *ced-5*, which encodes a DOCK180-like guanine exchange factor for Rac (Wu and Horvitz, 1998), and *hmr-1*, which encodes a classical cadherin (Costa et al., 1998), function redundantly in *C. elegans* gastrulation (Roh-Johnson et al., unpublished). *hmr-1* also contributes redundantly with *sax-7*, which encodes an L1CAM (Grana et al., 2010). We confirmed that Ea/Ep internalize successfully after injection of *hmr-1* dsRNA in a non-sensitized background or in a likely null allele of *ced-5*, *n1812* (Wu and Horvitz, 1998). However, gastrulation is often delayed, with the E cells internalizing as 4 cells, 25% of the time in

the double *hmr-1(RNAi)*; *ced-5(n1812)* (n=64) or 26% in *hmr-1(RNAi)*; *ced-5(RNAi)* embryos (n=23) (Roh-Johnson et al., unpublished). *hmr-1* appears to be relatively unique in this enhancement, as RNAi to several other putative adhesion genes (*rig-6*, *ncam-1*, *igcm-1*, and *byn-1*) and cytoskeletal regulators (*adm-2*, *pld-1*, *afd-1*, and *ced-2*) did not similarly enhance *ced-5(n1812)* (Figure 2.1). This result suggests that *ced-5(n1812)* sensitizes worms to depletion of specific genes, but does not overly sensitize them to depletion of all similar genes. Mutations in other Rac signaling components, *ced-2*/Crk, *ced-12*/ELMO and *ced-10*/Rac, were similarly enhanced by *hmr-1(RNAi)*, suggesting a redundant role for Rac signaling more generally in gastrulation (Figure 2.1).

We next determined if *ced-5(n1812)* would also be suitable as a sensitized background for feeding RNAi, by feeding bacteria expressing *GFP* dsRNA and *hmr-1* dsRNA to *ced-5(n1812)* worms and wild-type worms. The single treatments had low lethality: *ced-5(n1812)* had an embryonic lethality of 6%, and feeding *hmr-1* dsRNA to wild-type worms resulted in 8% embryonic lethality (Figure 2.2). Feeding *hmr-1* dsRNA to *ced-5(n1812)* mutant mothers resulted in 20% embryonic lethality (Figure 2.13), a significant enhancement (P=0.017).

To test whether the two useful backgrounds above might be combined to create a doubly-sensitized strain. We constructed a *ced-5(n1812);end-3(ok1448)* double mutant, and found that it had only 6% embryonic lethality, similar to the lethality of the single alleles (Figure 2.2), consistent with *ced-5* and *end-3* being in the same pathway and/or each being redundant with one or more other pathways. We reasoned that this low level of background lethality would facilitate detecting enhancement of lethality in an RNAi feeding screen, and that including both mutations in a sensitized strain might enable more

genes to be identified in the screen than including only one or the other, particularly if multiple, partially redundant mechanisms contribute to gastrulation, as has been predicted for morphogenesis more generally (Newman and Comper, 1990; Wieschaus, 1997). We found that the double mutant could be maintained as a homozygote, and that it retained the ability to be enhanced by feeding *mom-5* dsRNA or *hmr-1* dsRNA, as expected (Figure 2.3). Therefore, this strain was selected as our background to screen by RNAi for new genes with possible roles in gastrulation. After screening, we confirmed the value of the double mutant, which identified some enhancers that failed to significantly enhance one or the other of the single mutants (see below).

Identification of enhancers of the sensitized background among genes likely to be expressed in or near gastrulating cells

Our results above suggested that we would need to carefully quantify the degree of embryonic lethality for each treatment to identify enhancers. Therefore, to focus our effort, we selected a set of genes to screen through, making use of two previously published data sets that are likely to be enriched for genes expressed in the endodermal lineage or in their close neighbors from the MS lineage before or during gastrulation. First, the results of a published microarray expression experiment using precisely-timed embryos (Baugh et al., 2005) were re-ordered for us by L. R. Baugh (personal communication) to identify those genes whose mRNA abundances were higher in wild-type embryos than in *mex-3(zu155)*; *skn-1(RNAi)*. Embryos of this background generally lack properly specified E and MS lineages at the time when Ea and Ep would normally internalize, and, as expected, early endodermally-expressed mRNAs fail to accumulate (Baugh et al., 2005). We narrowed this list by the following criteria: first, we included

only those genes for which mRNA abundance rose by the time that Ea/Ep cell internalization occurred, using the microarray expression profiles of known endodermal genes to choose the relevant timepoints (Baugh et al., 2005), 23-101 min after the 4-cell stage. Second, we required mRNA abundances to be higher in wild-type embryos than in *mex-3(zu155)*; *skn-1(RNAi)* at these timepoints. Third, we also required mRNA abundances to be lower at these timepoints in wild-type embryos than in *pie-1(zu154)*; *pal-1(RNAi)*, a background where twice as many E and MS lineages form. The second list we used included a set of 50 genes identified in a microarray experiment designed to find early embryonic downstream targets of *skn-1*, called *sdz* (*skn-1*-dependent zygotic) genes, several of which are transcriptionally active in only MS and E descendents (Robertson et al., 2004). For convenience, we refer to both sets together as *sdz* genes, although *skn-1* dependence has not been validated for all of the genes included. Among these two sets, 112 clones existed in an RNAi feeding library (Kamath et al., 2001).

To assess the ability of knockdown of these 112 genes to enhance the gastrulation-sensitized strain, we fed these 112 bacterial feeding strains to the *ced-5;end-3* worm strain and to N2 wild-type worms in parallel for 48 hours. We assessed the resulting embryonic lethality by counting unhatched embryos and hatched worms at least 24 hours after removing adults (see Materials and Methods). After the first round of feeding, we repeated the top 70 results, as determined by enhancement of lethality, twice more. We found 22 genes that enhanced above an arbitrary threshold of 8% enhancement of lethality. Among these 22 genes, we identified *end-1*, which is already known to function redundantly with *end-3* in the E lineage as gastrulation begins (Maduro et al., 2005), validating the effectiveness of the screening method.

Before secondary screening, we tested whether screening in the double mutant background increased screening efficiency as predicted, by addressing whether synergy with *ced-5*, *end-3*, or both was responsible for the enhancements in lethality. We fed dsRNAs for the 22 genes identified, as well as for the positive controls *mom-5* and *hmr-1*, into the *ced-5* and *end-3* mutants separately (Figure 2.4). We found that loss of any of 15 genes enhanced significantly only in *ced-5* and none enhanced only in the *end-3* background. Three genes enhanced both *ced-5* and *end-3* backgrounds, including *end-1* and *mom-5*. Importantly, there were three genes that enhanced the double mutant but did not significantly enhance either of the single mutants, suggesting that the double mutant served as a more efficient sensitized background than either single mutant. Furthermore, these results begin to suggest a structure to the redundancy, which we plan to explore more fully in the future using null mutants.

Secondary screening implicates fifteen new genes in gastrulation

To identify which of these 22 genes were required for the normal pattern of gastrulation, we conducted a series of secondary screens. First, we injected dsRNAs targeting each gene into the endodermal GFP reporter strain JJ1317 *zuIs3* [*end-1*::GFP] (we hence refer to this as $P_{end\text{-}1}$::GFP) and filmed gastrulation in resulting embryos by 4D DIC microscopy (Thomas et al., 1996). The $P_{end\text{-}1}$::GFP strain served as a marker of endodermal fate, and we also assessed a later endodermal marker, birefringent gut granules (see Materials and Methods). We also injected each dsRNA into *ced-5(n1812)*, to determine the proportion of genes that affect gastrulation in this background. For many of the genes identified in our primary screen (20/22), including *end-1*, injection of dsRNA into *ced-5(n1812)* resulted in gastrulation defects (Figure 2.5). The number of

enhancers of *ced-5* found by dsRNA injection here and by dsRNA feeding above might reflect an especially effective sensitization for gastrulation genes by *ced-5(n1812),* or a role for *ced-5* in parallel to a large number of genes, or a combination of these possibilities. We also considered whether *ced-5(n1812)* overly sensitized the primary screen, revealing genes with only marginal roles in gastrulation, i.e. roles that could not be confirmed in a non-sensitized background. This appeared to not be the case: we identified 10 genes for which injection of dsRNA resulted in gastrulation defects in at least some embryos even in the non-sensitized strain P_{end-1}::GFP (Figure 2.5). Second, to examine possible stronger loss of function and to confirm our RNAi results with true mutants, we also filmed by 4D DIC microscopy mutants that were available for 12 of the 22 genes identified in the primary screen. For 10 of these 12 genes, we found that gastrulation defects occurred in the filmed mutant embryos (Figure 2.6). Most of these genes were named previously based on their sequence or as sdz genes. One of the genes, *glo-3*, encodes a novel protein that is expressed specifically in endoderm progenitors as early as the 2E cell stage (Rabbitts et al, 2008). Two of the genes were not previously named; we designate C10A4.5 and B0222.9 as *gad-2* and *gad-3*, respectively.

Because our starting list of 112 genes might already be enriched for genes involved in gastrulation, we further tested the value of our enhancer screen strategy by comparing it to a more commonly used method, a screen for embryonic lethality in *eri-1(mg366)*, a background with increased RNAi efficacy (Kennedy et al., 2004). Into *eri-1(mg366)* and wild-type worms, we fed bacterially-expressed dsRNAs targeting the 70 candidate genes we had screened in triplicate in *ced-5;end-3*, and quantified the degree of embryonic lethality (Figure 2.7). Among the 22 genes with the most penetrant embryonic

lethality in the *eri-1* background, five had been identified using *ced-5;end-3*. For the remaining 17, we injected dsRNAs into p_{end-1}::GFP animals and filmed resulting embryos by 4D DIC microscopy, quantifying gastrulation defects in these as before. This approach identified just two more gene with a very low penetrance, non-redundant role in gastrulation, and six more genes with a redundant role in gastrulation (Figure 2.8).

Taken together, these methods implicated 29 new genes in successful and timely gastrulation in *C. elegans*. Mutants or RNAi knockdown of 16 of these genes resulted in gastrulation defects, albeit subtle, in a non-sensitized background. Interestingly, *end-1* was not implicated in gastrulation by either RNAi of *end-1* in wild-type embryos nor by *end-1* deletion allele, suggesting that an earlier report of a role for *end-1* based on a larger deletion, wDf4, is likely explained by simultaneous deletion of *end-3* as well (Maduro et al., 2005; Lee et al., 2006). *end-1(RNAi)* did enhance gastrulation defects in the *ced-5* background, whereas *end-3(ok1446*) did not, raising the possibility that *end-3* and *ced-5* function in the same pathway, a model additionally supported by an enhancement of the subtle defects in *end-3(ok1446*) to stronger defects by injection *hmr-1*dsRNA (increased to 55% from 0% stronger defects). However, further pathway analysis is something we will pursue in the future with null mutants. Six of the 23 genes we identified had quite low penetrance effects on gastrulation and a higher penetrance in *ced-5(n1812)*, while 13 others could only be implicated in combination with *ced-5(n1812)*, suggesting that many of these genes may act redundantly or partially redundantly in gastrulation, or indirectly in processes contributing to normal gastrulation.

Several of the newly identified genes belong to gene families arising evolutionary gene duplication

BLAST analysis of our newly identified genes indicated that for many of these genes (12/15), the closest known sequence as judged by BLAST score in the NCBI nr database as of Sept 2010 was another gene in the *C. elegans* genome. For a large proportion of the genes (11/15), multiple other *C. elegans* genes had higher BLAST scores than did any non-nematode or even non-*Caenorhabditis* genes. We hypothesize that either a) many of these genes belong to gene families that arose from rounds of gene duplication events within the nematode lineage, or b) represent a large set of convergently evolved genes. Since *C. elegans* has a compact genome with mostly single copy genes (Woollard, 2005), our screen appeared to have enriched for such genes.

C. elegans gene families deriving from recent gene duplications are more likely to function redundantly than are single copy genes (Conant and Wagner, 2003), and we speculate that this is true for sets of similar genes deriving from less recent duplications or convergent evolution as well. Therefore, given the subtle defects and low penetrance of many of the fifteen genes we identified, and our finding of eight genes that could only be identified in sensitized backgrounds, we hypothesize that our screening method was successful in uncovering genes that function redundantly or partially redundantly in *C. elegans* gastrulation. We tested this hypothesis directly for one gene family below.

***GADR-1*, a redundant gastrulation defective gene expressed at gastrulation onset**

One of the most penetrant enhancers of *ced-5(n1812)* lethality that we found was loss of F58D2.1 (Figure 2.4). F58D2.1 acted synergistically with *ced-5* in gastrulation: targeting F58D2.1 and *ced-5* together, by injecting F58D2.1 dsRNA into *ced-5(n1812)* worms, resulted in 25% of embryos failing in Ea/Ep internalization, whereas neither

single mutant clone produced this result (Figures 2.1 and 2.5). Based on this result and others below, we name *F58D2.1, gadr-1* (gastrulation defective, redundant gene).

Microarray experiments on staged embryos (Baugh et al., 2005) demonstrated that *gadr-1* transcript abundance increased near the time that gastrulation begins -- soon after *end-1* transcripts, which are first detected in the E cell by *in situ* hybridization (Zhu et al., 1997), and before *elt-2* transcripts, which are first detected in Ea and Ep just after gastrulation begins (Fukushige et al., 1998). To determine when and where the GADR-1 protein accumulates, we generated an affinity-purified rabbit antibody to a 100 residue protein fragement (see Materials and Methods) and used this antibody to immunostain embryos. By immunostaining, timing was consistent with the microarray results and with our proposed role in gastrulation: GADR-1 immunoreactivity became strong during endodermal internalization. GADR-1 immunoreactivity localized to both nuclei and cytoplasm of all cells, with a small amount of enrichment near cell-cell boundaries (Figure 2.9). This pattern was eliminated by *gadr-1 (RNAi),* or by a deletion allele, *ced-3(n2452),* which is a 17kb deletion that removes all or parts of six genes including most of *gadr-1* (Shaham et al., 1999) and the entire antigen sequence. In support of our hypothesis from RNAi experiments that *gadr-1* functions redundantly in gastrulation, this deletion allele produced gastrulation defects only in combination with *ced-5(RNAi),* and not alone (Figure 2.1). We conclude that *gadr-1* functions redundantly in gastrulation, and that it encodes a nuclear and cytoplasmic protein that is first expressed in all cells near the time that gastrulation begins.

GADR-1 and paralogs are the result of the expansion of a gene family related to ZYG-11

A search for similar genes by BLAST identified the predicted GADR-1 protein as belonging to a large and diverse group of *C. elegans* proteins that includes ZYG-11, which is a substrate recognition subunit for a CUL-2 cullin ubiquitin ligase complex (Vasudevan et al., 2007) and ZEEL-1, a related protein implicated in reproductive incompatibility between populations (Seidel et al., 2008). By BLAST of the predicted GADR-1 protein sequence, 23 predicted *C. elegans* proteins had lower E values than any non-nematode sequence in the nr database, suggesting that these genes may have arisen from rounds of gene duplication within nematodes, or that they arose from convergent sequence evolution.

We used phylogenetic and comparative genomic analysis to reveal the evolutionary history of the newly identified genes relative to *C. elegans vhl-1*, *zif-1*, *zer-1*, and *zyg-11*, and human and mouse ZYG-11 homologs. These highly diverged amino acid sequences produced a star phylogeny with the exception of several sets of genes within *C. elegans* and the mammalian ZYG-11 gene family (Figures 2.10). Outside of the mammalian clade, which resolves as expected, only three clades form monophyletic groups (Figure 2.10A-C) with significant bootstrap support using both the maximum likelihood (ML) and maximum parsimony (MP) methods (the clade with *zyg-11* and F47D12.5 is not supported with MP). The paralogs in Clades A and C are highly divergent, suggesting an ancient origin. Comparison to *C. briggsae* shows that the genes Clades A and C have a single *C. briggsae* homolog (CBG07183 and CBG24348, respectively). Thus, the duplications within Clades A and C likely occurred after the split of *C. elegans* from *C. briggsae* 100 MYA. The paralogs within Clade B show a classic pattern of repeated rounds of duplication within the *C. elegans* lineage. The evolutionary

timing of these duplications is less clear. Y71A12B.12a and F47G4.2 share about 54% amino acid sequence identity for the homologous regions (Y71A12B.12a has 15 additional residues). Both genes share about 42% identity with *C. briggsae* CBG24348. However, F47G4.2 is equally similar to CBG07183. Indeed the majority of the newly identified genes show greatest similarity to either CBG07183 or CBG24348 in *C. briggsae*. CBG07183 and CBG24348 are somewhat similar to *C. briggsae* ZYG-11 (25% identity, 45% similarity for aligned regions). This observation suggests that many of the newly identified genes in our *C. elegans* screen may have diversified since the split with *C. briggsae*. The high amino acid divergence among these sequences results in poor resolution of the phylogeny, which prevents a direct test of this hypothesis.

Sequence similarity among F58D2.1 and paralogs is driven by a small set of residues corresponding to Leucine-Rich-Repeats (LRR) and several uncharacterized motifs

Our observation that newly identified genes appear to have higher sequence similarity with other genes supports our model that these genes tend to be functionally redundant. This model also suggests that these genes should share common features and motifs. We performed a comparative sequence analysis of the newly identified genes, members of the *zyg-11* family, *vhl-1*, *zif-1*, and *zer-1*. Using the Conserved Domains Database, we noticed that all genes analyzed including the mammalian zyg11 genes had at least one leucine-rich-repeat like motif (canonically, LxxLxLxxN/CxL). In our trimmed conserved alignment leucine residues are enriched across taxa from position 144 to 429. There are several shared motifs specific to newly identified genes as well as shared sites within motifs shared among most of the genes (data not shown). While most of these protein sequences are highly divergent, the strong similarity within these specific

motifs in the newly identified genes suggests that these motifs are evolutionarily and functionally conserved. As functional conservation often results in redundancy, these data support our belief that the newly identified genes are likely functionally redundant.

***gadr-1* to *-6* act redundantly with each other during gastrulation**

We hypothesized that *gadr-1* functions redundantly in gastrulation with one or more genes showing sequence similarity. To identify such genes, we injected dsRNA targeting the 9 closest relatives of *gadr-1* by BLAST into both *ced-5(n1812)* and p_{end-1}::GFP worms. We found that most of these could enhance *ced-5(n1812)* lethality, but none produced gastrulation defects in the non-sensitized background, P_{end-1}::GFP, suggesting that all of these genes act redundantly, as *gadr-1* does (Table 1). Indeed, one of these genes, C48D1.1, is also entirely absent in the *n2452* deletion allele described above. This result implied that if *gadr-1* contributes redundantly to gastrulation with some of the related genes, deleting just this pair was not sufficient to reveal a gastrulation defect.

We pursued our hypothesis of redundancy by pooled injection of dsRNAs with the other related genes. Loss of both C48D1.1 and F53G2.1 conferred frequent cell division defects before gastrulation in *ced-5(n1812)* and were not pursued further. Injection of pooled dsRNAs targeting six remaining genes (the six with the most penetrant effects on gastrulation in *ced-5(n1812)*) into N2 worms resulted in 49% penetrant gastrulation defects in Ea/Ep cell internalization (27/55 embryos) (Table 2). This result confirms that some or all of these six related genes function redundantly with each other in one or more processes that directly or indirectly affect gastrulation.

To elucidate whether some play more significant roles than others in gastrulation, we used a strategy of injecting all combinations of five of the six pooled dsRNAs, then omitting the one that gave the least penetrant gastrulation defects in a following round using pools of four dsRNAs, and reiterating this pattern until we had narrowed down to just a pair of genes with the most penetrant effects (Table 2). We found that decreasing the number of genes decreased the penetrance of the phenotypes at nearly every step, without any genes emerging as especially major contributors (Table 2). This result suggests that these genes function partially redundantly in an additive manner with one another (Table 2). We conclude that each of these genes (which we designate *gadr-2* (C33A12.12), *gadr-3* (F47D12.5), *gadr-4* (W06A11.2), *gadr-5* (Y71A12B.17) and *gadr-6* (F47G4.2)) acts redundantly with *ced-5* in gastrulation, and that all or most of them act redundantly with each other in gastrulation. Our results indicate that our strategy for identifying new gastrulation genes can successfully identify redundant players, including sets of related genes that function redundantly with each other.

***gadr-1* to -*6* may be SRSs for CUL-2 ubiquitin ligase complexes**

The observation that the GADR-1 to -6 gene family is related to ZYG-11, a substrate recognition subunit (SRS) for a CUL-2 ubiquitin ligase complex, suggested that these proteins function similarly to SRSs in CUL-2 complexes. Affinity purifications coupled to liquid chromatography and tandem mass spectrometry (LC-MS/MS) were used to identify proteins that physically associate with CUL-2::FLAG *in vivo*. In two separate samples, GADR-6/F47G4.2 was identified in affinity purifications from lysates of animals expressing CUL-2::FLAG. The number of peptides of GADR-6 identified by LC-MS/MS in the two samples (9 and 11 peptides) was comparable to the number of

peptides observed for known SRSs: FEM-1, 24 and 32 peptides; ZER-1, 19 and 29; ZYG-11, 9 and 11; LRR-1, 3 and 5; VHL-1, 0 and 0; and ZIF-1, 0 and 0. The other members of the extended GADR-1 family were not identified in the affinity purifications. However, in separate affinity purifications that only analyzed the 85-140 kDa region on SDS-PAGE gels, GADR-5/Y71A12B.17 was identified by a single peptide in the CUL-2::FLAG purification sample (while GADR-6 was identified with 4 peptides; ZYG-11, 8 peptides; and ZER-1, 12 peptides); none of these proteins was identified from the comparable 85-140 kDa region of the control affinity purification (from wild-type animals not expressing CUL-2::FLAG).

To further probe if GADR-5 and GADR-6 function as SRSs, we asked whether they could interact with CUL-2 and the CUL-2-complex adaptor protein Elongin C/ELC-1 when ectopically expressed in HEK293T human cells. We observed that CUL-2 and ELC-1 co-immunoprecipitated with GADR-5 and GADR-6 at a level comparable to that observed with ZYG-11 immunoprecipitation (Figure 2.16). Therefore, GADR-5 and GADR-6 are likely candidates to be SRSs for CUL-2 ubiquitin ligase complexes. The failure to detect other GADR-1 paralogs in affinity purifications of CUL-2::FLAG may be due to the limitations of the analysis, as the affinity purification coupled to LC-MS/MS approach also failed to identify the previously identified SRSs VHL-1 and ZIF-1.

DISCUSSION

Redundancy has been proposed to be a well-recognized aspect of morphogenesis, making gene discovery a challenge (Newman and Comper, 1990; Wieschaus, 1997). We decided to address this problem directly using both classical genetics and RNAi while looking for new genes acting in *C. elegans* gastrulation. In this paper, we have described an enhancer screen to find new *C. elegans* gastrulation genes, the first RNAi modifier screen for gastrulation genes in *C. elegans*. We find that there is indeed developmental redundancy both between similar genes and between genes that are unrelated by sequence -- homologous and non-homologous redundancy (Jorgensen and Mango, 2002). We also observed that several genes found to have a role in *C. elegans* gastrulation belong to groups of related genes, some of which may represent gene families deriving from gene duplication events in the nematodes. We predicted that such genes may be more likely than single copy genes to function redundantly or partially redundantly, and we confirmed this for one set of six related genes, *gadr-1* to *-6*. Our results demonstrate that screening by RNAi in a sensitized background is a viable method for tackling redundancy, and that it can even identify redundant, closely related genes, traditionally thought of as difficult to identify genetically.

Using RNAi to screen for genes involved in morphogenetic processes

Many *C. elegans* biologists have taken advantage of the ease of RNAi to compile relatively quickly a list of genes involved in a process of interest (reviewed in Jorgensen and Mango, 2002 and Boutros and Ahringer, 2008). With speed and ease of methodology

comes the drawback of variable and sometimes ineffective RNAi, especially when using feeding RNAi as opposed to RNAi by injection. Even with these drawbacks, an RNAi screen can be valuable in tackling redundancy and studying somewhat genetically refractory developmental processes.

Often, suppressor screens (Labbé et al., 2006; O'Rourke et al., 2007; Dorfman et al., 2009; reviewed in Boutros and Ahringer 2008) have been utilized to discover new genes that function in early developmental processes. The ability to screen for survivors starting from a conditional lethal strain is rapid and convenient. To screen for enhancers, or to do so efficiently, one must be able to recognize quickly the enhanced phenotypes. In our case, we sensitized our worms using mutations known to affect gastrulation and used embryonic lethality as a first pass test for enhancement. We then used 4-D microscopy to examine the initiation of gastrulation, internalization of the E cells.

One goal of our screen was to identify new genes whose functions are required for normal gastrulation. Although this succeeded, limitations exist in the screen that we have carried out. Filming embryos revealed many low penetrance gastrulation genes, and it is possible that we may have missed other genes whose loss of function in wild-type embryos may produce similar defects, but that would have been missed if they did not significantly increase lethality of the sensitized background used in our primary screen. We also did not explore defects in developmental processes other than Ea/Ep internalization. Therefore, defects in later morphogenesis or other processes could be a separate cause of enhancement of lethality from our primary screen. We started with a candidate set of zygotic genes, introducing the possibility that we have missed some important maternal genes. We expect that the genes we have identified

may include genes that affect gastrulation either directly or indirectly. At least one is expressed in Ea and Ep, suggesting a more direct role than is likely with *gadr-1*, which we have shown is expressed near the time of gastrulation, but in all cells. The *sdz* gene set is likely to be enriched for genes expressed specifically in the E and/or MS lineages (Robertson et al., 2004). The genes we have identified probably represent only a small proportion of all genes that function in gastrulation, though what proportion is difficult to estimate.

Non-homologous genetic redundancies have been found in *C. elegans* before (e.g. Culotti et al. 1981; Johnson et al. 1981; Ferguson and Horvitz 1989; Davies et al. 1999). One well characterized *C. elegans* non-homologous redundancy is the synthetic multi-vulval (SynMuv) genes (Ferguson et al. 1987; Ferguson and Horvitz 1989; for review, see Fay and Han 2000; Fay, et al., 2002). We identified several genes that could only be implicated in gastrulation in specific genetic backgrounds, and not in wild-type worms. We refer to such a synthetic gastrulation phenotype as SynGad. We look forward to the further exploration of how genes with SynGad phenotypes regulate the processes that contribute to gastrulation.

Predicted roles for some of the new genes involved in *C. elegans* gastrulation

Many of the genes we have identified encode proteins of unknown function in *C. elegans* but have specific, predicted protein domains (Table 3). For example, *tbx-11* encodes a putative T-box transcription factor of the Tbx2 subfamily, and a function for *tbx-11* had not been reported previously. We have found that depletion of *tbx-11* did not detectably perturb the E lineage marker p_{end-1}::GFP, nor the MS lineage markers *tbx-*

35::GFP or *dlx-1*::GFP (Table 3 and data not shown), making the function of *tbx-11* in gastrulation elusive thus far.

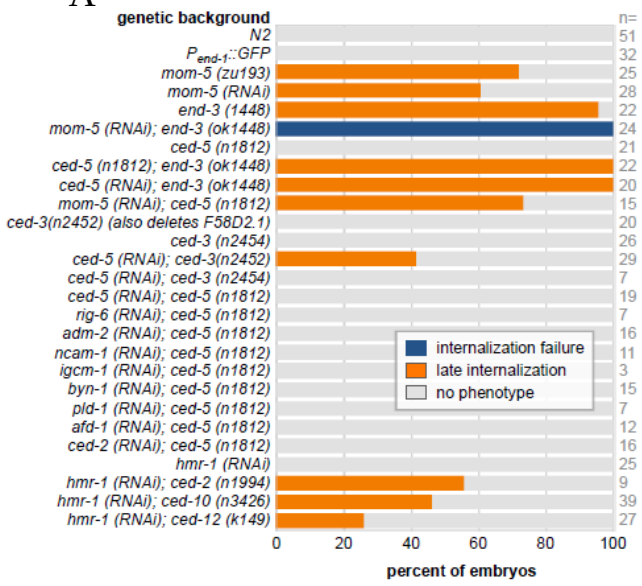
glo-3, which is expressed specifically in endoderm progenitors as early as the 2E cell stage, has been proposed to function later in vesicle trafficking to the embryonic gut granules (Rabbitts et al, 2008). GLO-3 protein is likely to play a direct role in regulating the formation, maturation, and/or stability of gut granules, since a rescuing GLO-3::GFP fusion is localized to the gut granule membrane. *apy-1* encodes a predicted apyrase, a membrane-bound enzyme that catalyzes the hydrolysis of nucleoside triphosphates and diphosphates. *apy-1* mutant worms abnormally accumulate intestinal autofluorescence, which has been interpreted as a lysosomal traffic defect also associated with aging (Uccelletti et al, 2008). Taken together, these results suggest the possibility that normal lysosomal trafficking might play a specific role in successful gastrulation, a prediction that will be tested by future experiments.

FIGURES

Figure 2.1

Enhancement of subtle gastrulation defects. (A) Bar graph representing the tabulated gastrulation defects in different genetic backgrounds, mutant alleles and injected dsRNAs, for both internalization failure, where the E cells remain on the surface of the embryo (dark blue), and late internalization, where the E cells divide on the surface and internalize as 4 E cells (orange). N values are shown on the right. (B) 4-D DIC microcopy of 4 different genetic backgrounds with time on the left from 2MS cell division. E cells are outlined and pseudocolored in green. Defective gastrulation is indicated by black arrowheads. Scale: *C. elegans* embryos are approximately 50µm long.

A


genetic background
n=
N2 51
Pend-1::GFP 32
mom-5 (zu193) 25
mom-5 (RNAi) 28
end-3 (1448) 22
mom-5 (RNAi); end-3 (ok1448) 24
ced-5 (n1812) 21
ced-5 (n1812); end-3 (ok1448) 22
ced-5 (RNAi); end-3 (ok1448) 20
mom-5 (RNAi); ced-5 (n1812) 15
ced-3(n2452) (also deletes F58D2.1) 20
ced-3 (n2454) 26
ced-5 (RNAi); ced-3(n2452) 29
ced-5 (RNAi); ced-3 (n2454) 7
ced-5 (RNAi); ced-5 (n1812) 19
rig-6 (RNAi); ced-5 (n1812) 7
adm-2 (RNAi); ced-5 (n1812) 16
ncam-1 (RNAi); ced-5 (n1812) 11
igcm-1 (RNAi); ced-5 (n1812) 3
byn-1 (RNAi); ced-5 (n1812) 15
pld-1 (RNAi); ced-5 (n1812) 7
afd-1 (RNAi); ced-5 (n1812) 12
ced-2 (RNAi); ced-5 (n1812) 16
hmr-1 (RNAi) 25
hmr-1 (RNAi); ced-2 (n1994) 9
hmr-1 (RNAi); ced-10 (n3426) 39
hmr-1 (RNAi); ced-12 (k149) 27
internalization failure
late internalization
no phenotype
0
20
40
60
80
100
percent of embryos

B

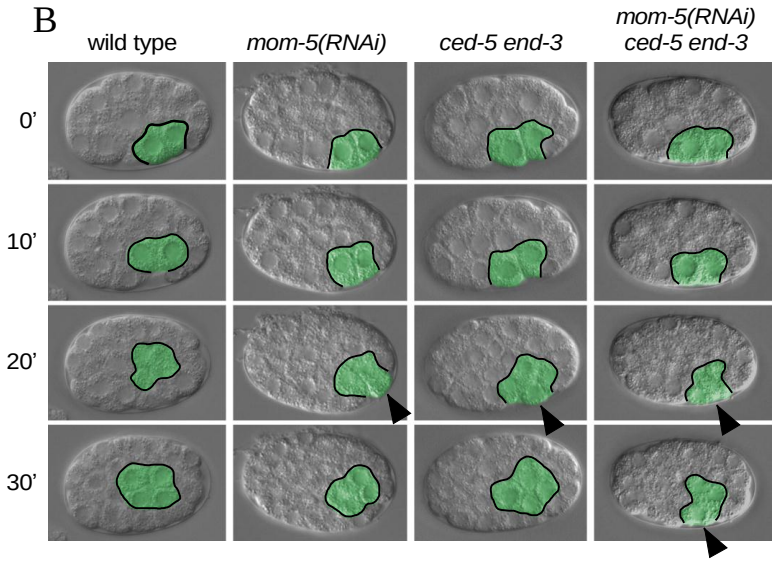

wild type
mom-5(RNAi)
ced-5 end-3
mom-5(RNAi) ced-5 end-3
0’
10’
20’
30’

Figure 2.2

ced-5(n1812);end-3(ok1448) double mutant has similar percent lethality as each single mutant. Percentage lethality was determined by feeding negative control bacterial strain, containing the plasmid L4440 expressing dsGFP, into *ced-5(n1812)* (red), *end-3(ok1448)* (blue), and *ced-5(n1812);end-3(ok1448)* (green). Error bars indicate 1 SE.

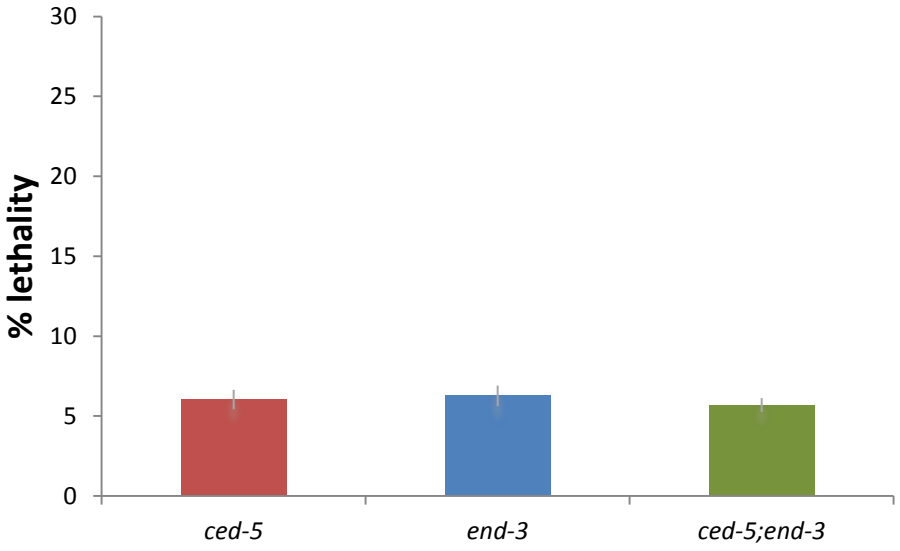


Figure 2.3

Primary screen feeding dsRNAs targeting sdz genes into the gastrulation-sensitized background. Percentage enhancement of lethality in gastrulation, i.e. lethality in the sensitized background, ced-5(n1812);end-3(ok1448) minus wild-type lethality (See Material and Methods).Only experimental pairs tested three times or more are shown. Dashed red line indicates an arbitrary threshold of 8% enhancement of lethality. Error bars indicate 1 SE.

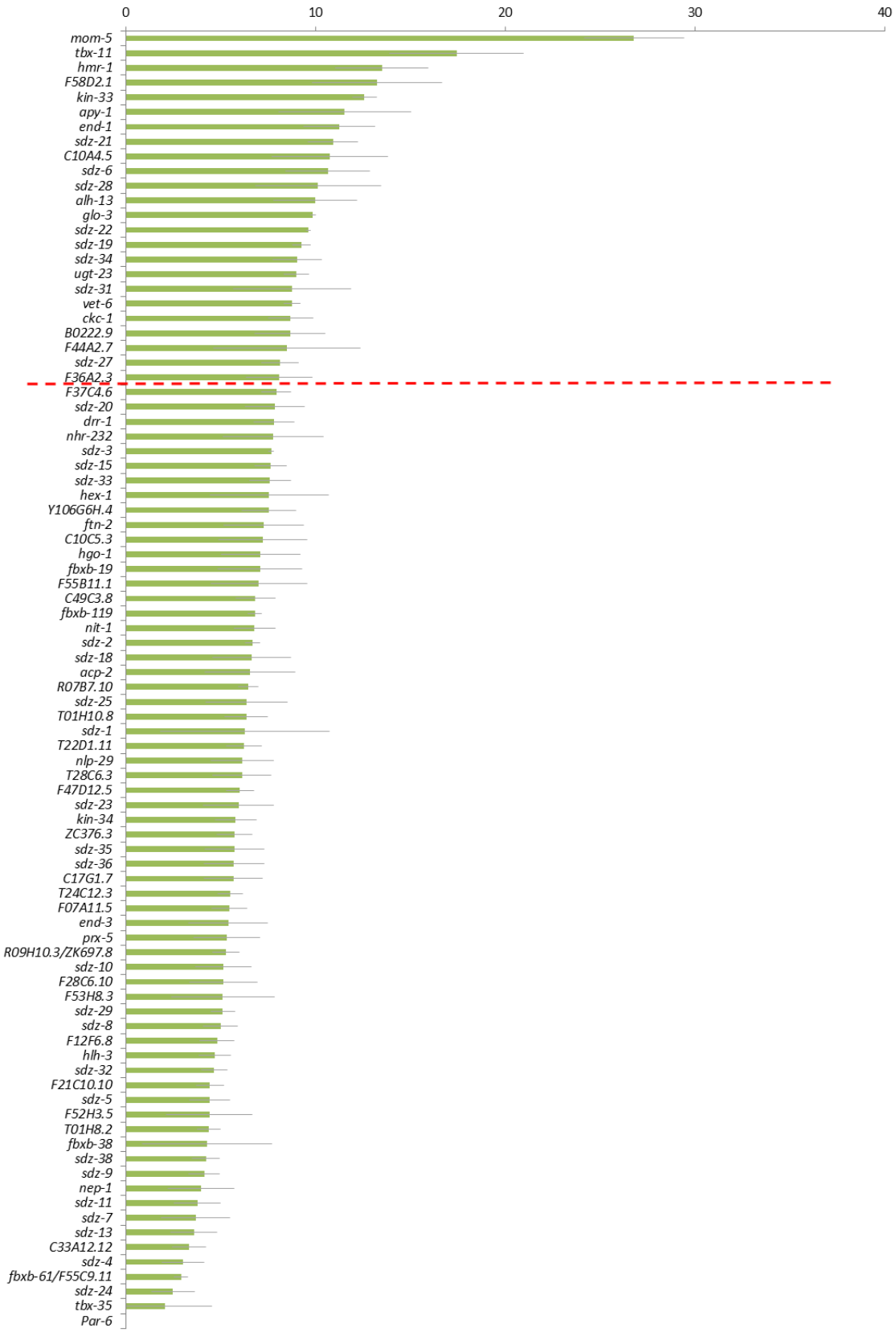

0
10
20
30
40
mom-5
tbx-11
hmr-1
F58D2.1
kin-33
apy-1
end-1
sdz-21
C10A4.5
sdz-6
sdz-28
alh-13
glo-3
sdz-22
sdz-19
sdz-34
ugt-23
sdz-31
vet-6
ckc-1
B0222.9
F44A2.7
sdz-27
F36A2.3
F37C4.6
sdz-20
drr-1
nhr-232
sdz-3
sdz-15
sdz-33
hex-1
Y106G6H.4
ftn-2
C10C5.3
hgo-1
fbxb-19
F55B11.1
C49C3.8
fbxb-119
nit-1
sdz-2
sdz-18
acp-2
R07B7.10
sdz-25
T01H10.8
sdz-1
T22D1.11
nlp-29
T28C6.3
F47D12.5
sdz-23
kin-34
ZC376.3
sdz-35
sdz-36
C17G1.7
T24C12.3
F07A11.5
end-3
prx-5
R09H10.3/ZK697.8
sdz-10
F28C6.10
F53H8.3
sdz-29
sdz-8
F12F6.8
hlh-3
sdz-32
F21C10.10
sdz-5
F52H3.5
T01H8.2
fbxb-38
sdz-38
sdz-9
nep-1
sdz-11
sdz-7
sdz-13
C33A12.12
sdz-4
fbxb-61/F55C9.11
sdz-24
tbx-35
Par-6

Figure 2.4

Specificity for enhancement of embryonic lethality into single components of sensitized background. (A) Percentage enhancement of lethality of *ced-5(n1812)* over wild-type lethality (red). (B) Percentage enhancement of lethality of *end-3(ok1448)* over wild-type lethality (blue). Error bars indicate 1 SE.

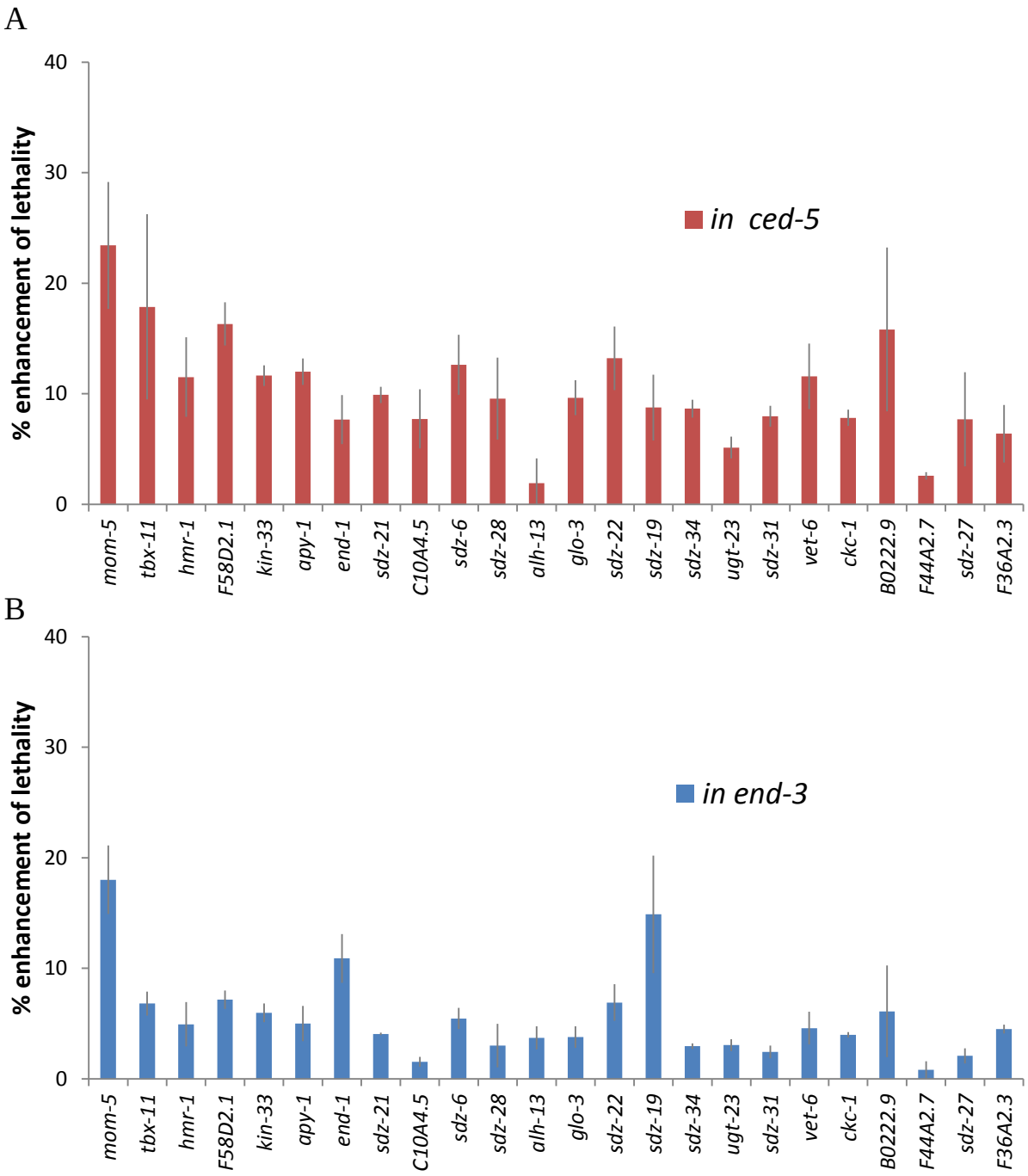

A
% enhancement of lethality
40
30
20
10
0
in ced-5
mom-5
tbx-11
hmr-1
F58D2.1
kin-33
apy-1
end-1
sdz-21
C10A4.5
sdz-6
sdz-28
alh-13
glo-3
sdz-22
sdz-19
sdz-34
ugt-23
sdz-31
vet-6
ckc-1
B0222.9
F44A2.7
sdz-27
F36A2.3
B
% enhancement of lethality
40
30
20
10
0
in end-3
mom-5
tbx-11
hmr-1
F58D2.1
kin-33
apy-1
end-1
sdz-21
C10A4.5
sdz-6
sdz-28
alh-13
glo-3
sdz-22
sdz-19
sdz-34
ugt-23
sdz-31
vet-6
ckc-1
B0222.9
F44A2.7
sdz-27
F36A2.3

Figure 2.5

New gastrulation genes from gastrulation-sensitized screen found by dsRNA injection. Bar graphs representing the tabulated gastrulation defects from injected dsRNAs into non-sensitized (N2 and P_{end-1}::GFP) and sensitized (*ced-5(n1812)*) backgrounds. Both internalization failure, where the E cells remain on the surface of the embryo (dark blue), and late internalization, where the E cells divide on the surface and internalize as 4 E cells (orange) are represented. N values are on the right of each bar graph.

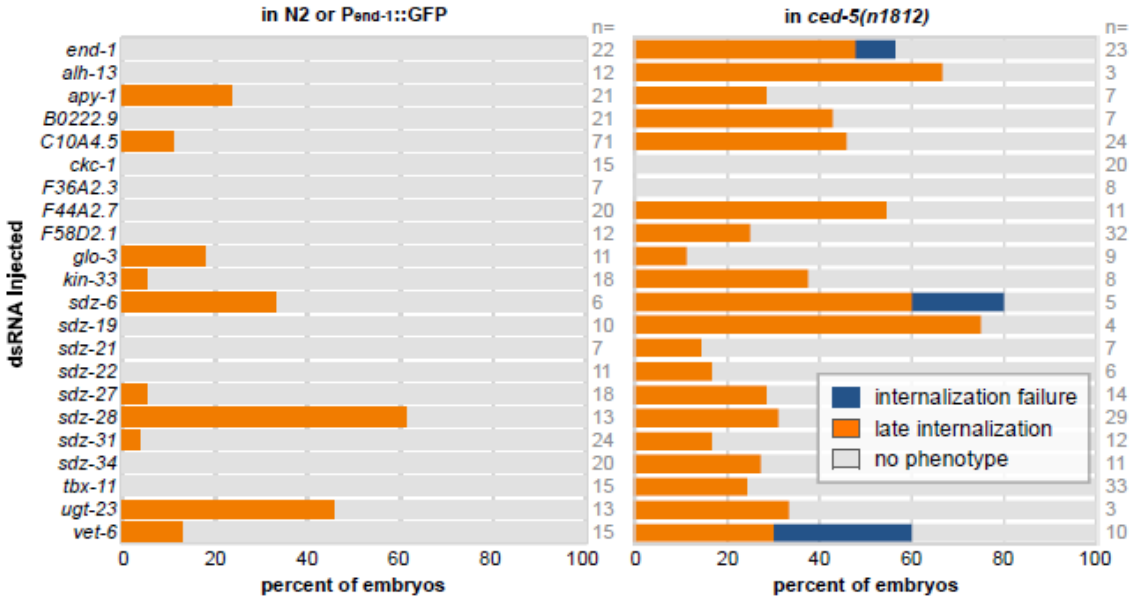


Figure 2.6

Mutants with subtle gastrulation defects. Bar graphs representing the tabulated gastrulation defects in mutant alleles. Subtle defects are defined as late internalization, where the E cells divide on the surface and internalize as 4 E cells (orange). N values are on the right.

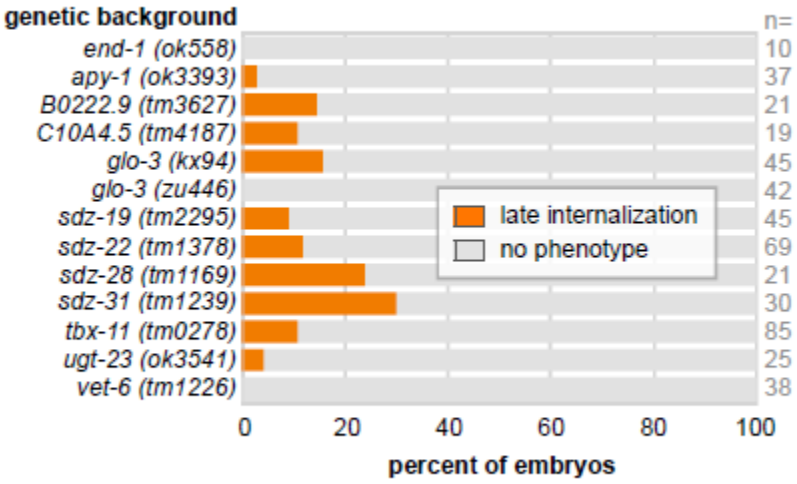


Figure 2.7

Embryonic lethality in an RNAi-sensitized background. Bar graph of the percent lethality resulting from feeding each dsRNA to an RNAi-sensitized, *eri-1(mg366)*(blue) or wild-type (red) background. Dashed red line indicates top 22 positive results. Error bars indicate 1 SE.

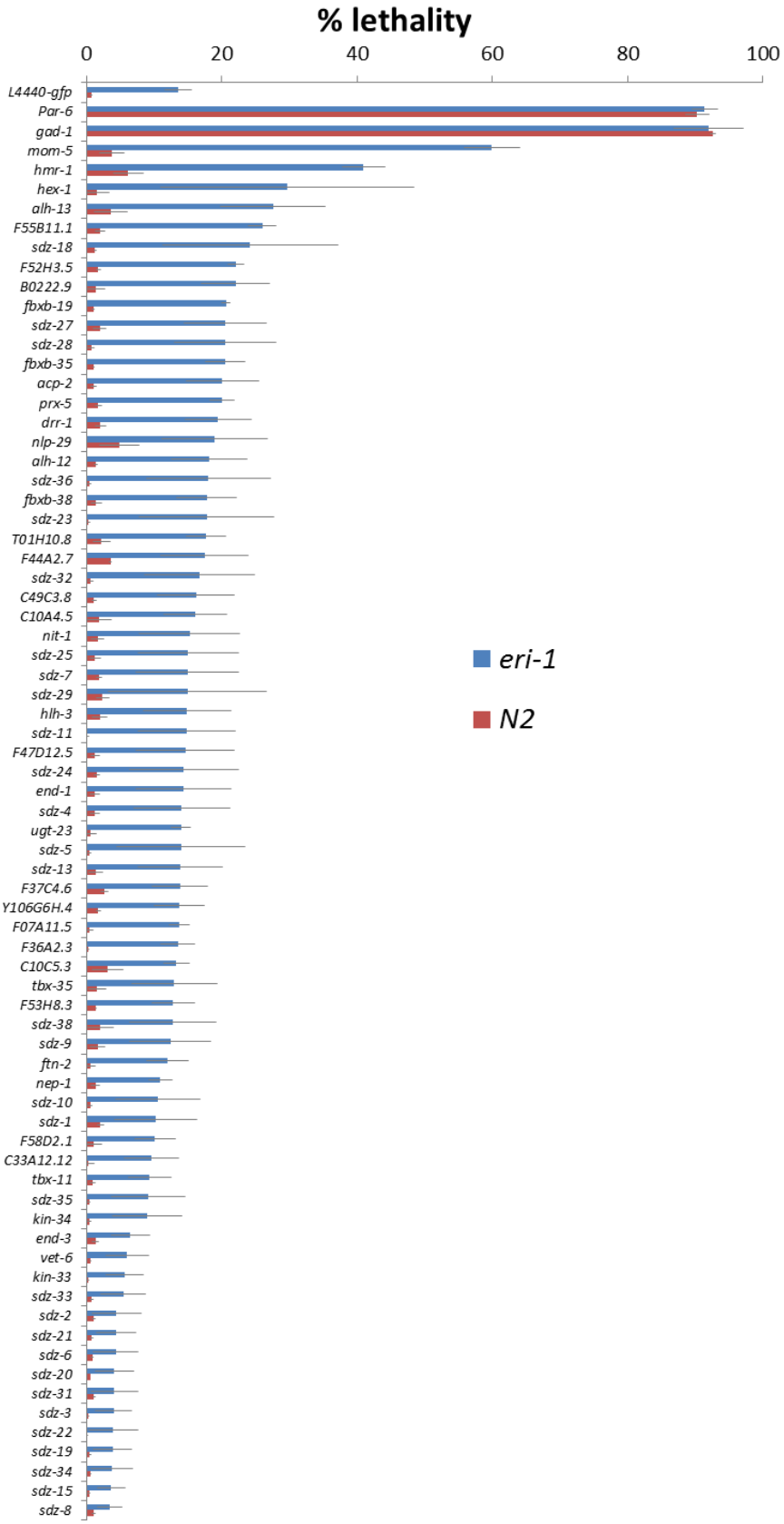

% lethality
0
20
40
60
80
100
L4440-gfp
Par-6
gad-1
mom-5
hmr-1
hex-1
alh-13
F55B11.1
sdz-18
F52H3.5
B0222.9
fbxb-19
sdz-27
sdz-28
fbxb-35
acp-2
prx-5
drr-1
nlp-29
alh-12
sdz-36
fbxb-38
sdz-23
T01H10.8
F44A2.7
sdz-32
C49C3.8
C10A4.5
nit-1
sdz-25
sdz-7
sdz-29
hlh-3
sdz-11
F47D12.5
sdz-24
end-1
sdz-4
ugt-23
sdz-5
sdz-13
F37C4.6
Y106G6H.4
F07A11.5
F36A2.3
C10C5.3
tbx-35
F53H8.3
sdz-38
sdz-9
ftn-2
nep-1
sdz-10
sdz-1
F58D2.1
C33A12.12
tbx-11
sdz-35
kin-34
end-3
vet-6
kin-33
sdz-33
sdz-2
sdz-21
sdz-6
sdz-20
sdz-31
sdz-3
sdz-22
sdz-19
sdz-34
sdz-15
sdz-8
eri-1
N2

Figure 2.8

New gastrulation genes from RNAi-sensitized screen found by dsRNA injection. Bar graphs representing the tabulated gastrulation defects from injected dsRNAs into non-sensitized (N2 and $P_{end\text{-}1}$::GFP) and sensitized (*ced-5(n1812)*) worms. N values are on the right of each bar graph.

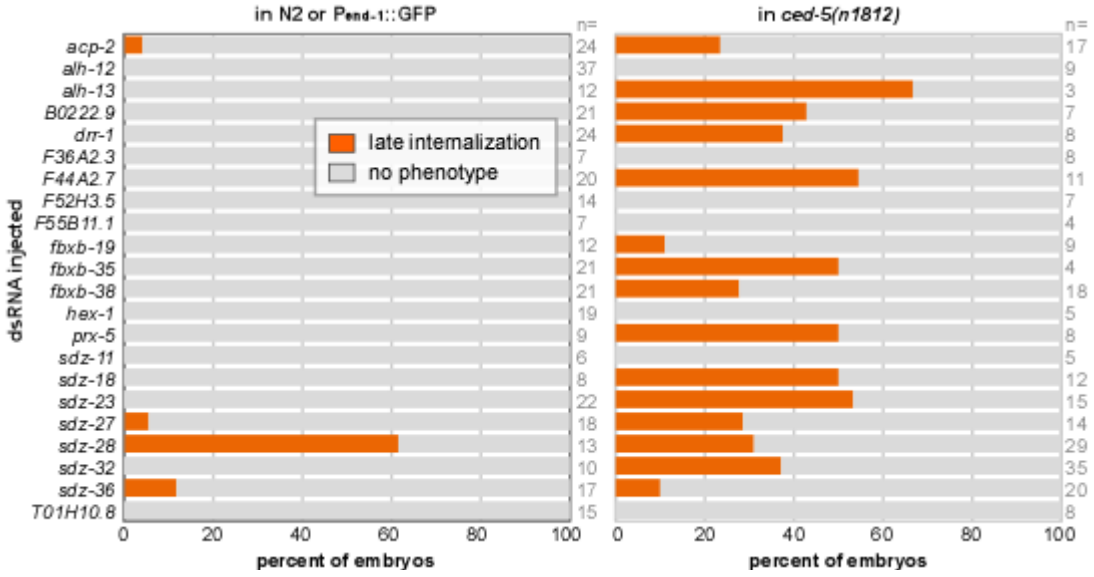


Figure 2.9

GADR-1 protein levels elevate prior to gastrulation. Wild-type embryos imaged from the same slide, under identical conditions with an antibody for GADR-1 (green) and DAPI (blue), are shown below. Early embryos (such as 4-cell embryo in A) have a decreased level of protein until prior to gastrulation (B). Presence of the protein is maintained after E cell internalization (C) and further into embryonic development (not shown).The antibody staining does seem to be specific, depletion of *gadr-1* by RNAi (D) or use of a large deletion mutant that includes the *gadr-1* gene (E), do not visualize any endogenous protein from gastrulating embryos. Scale: *C. elegans* embryos are approximately 50µm long.

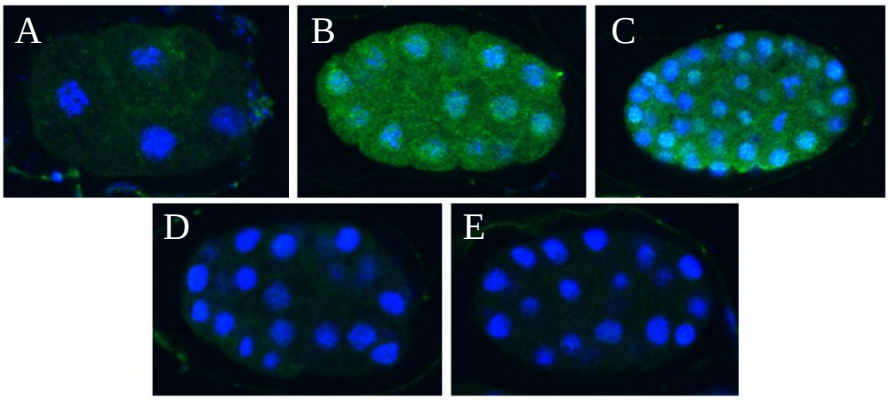


Figure 2.10

Phylogenetic relationship of the newly identified genes, related *C. elegans* genes, and mammalian zyg11 genes. We used both maximum likelihood and maximum parsimony to produce phylogenies of the newly identified genes, *C. elegans vhl-1*, *zif-1*, *zer-1*, and *zyg-11*, and human and mouse zyg11 homologs. These genes do not form a monophyletic group, although several distinct clades are supported (Clades A-D). Comparative analysis to *C. briggsae* shows that Clades A-C likely arose after the split of *C. elegans* and *C. briggsae*.

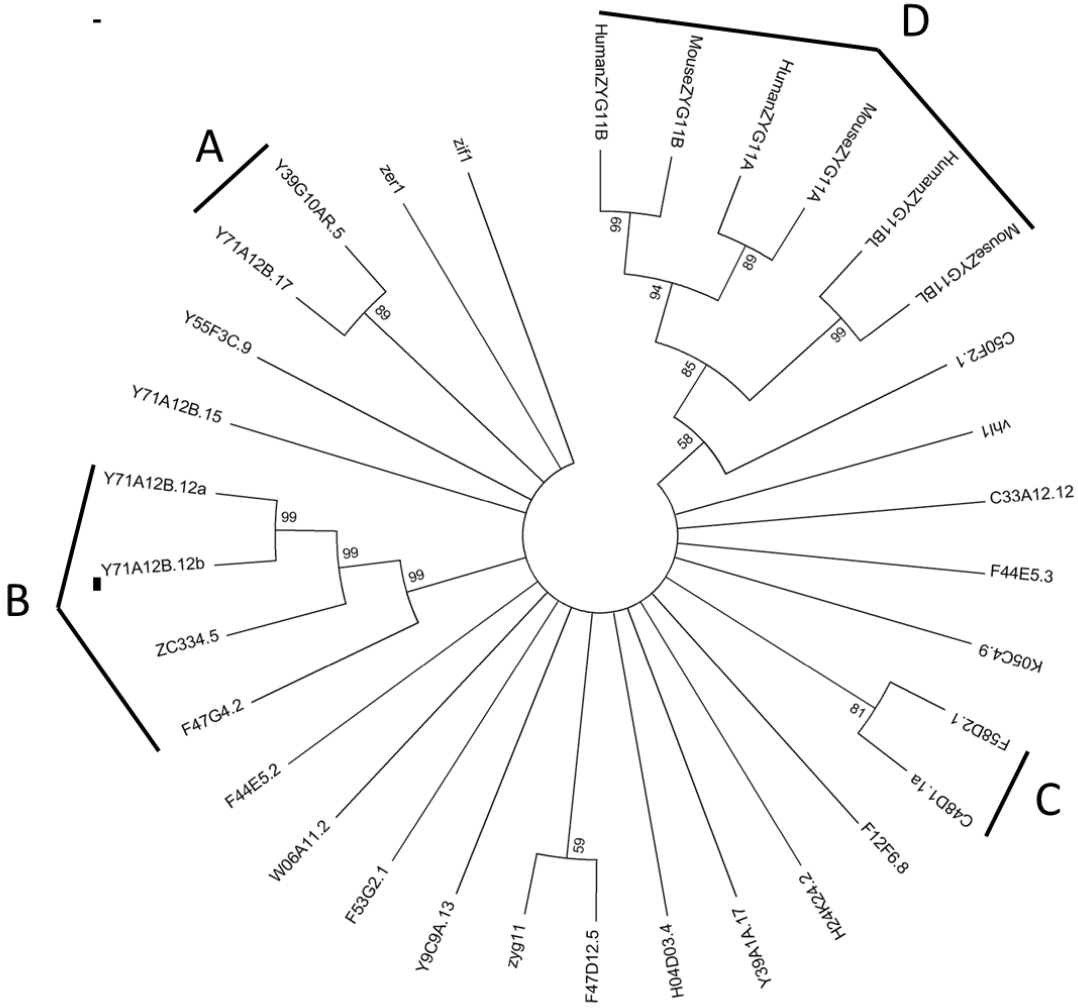

-
A
B
C
D
Y39G10AR.5
Y71A12B.17
Y55F3C.9
Y71A12B.15
Y71A12B.12a
Y71A12B.12b
ZC334.5
F47G4.2
F44E5.2
W06A11.2
F53G2.1
Y9C9A.13
zyg11
F47D12.5
H04D03.4
Y39A1A.17
H24K24.2
F12F6.8
C48D1.1a
F58D2.1
K05C4.9
F44E5.3
C33A12.12
vhl1
C50F2.1
MouseZYG11BL
HumanZYG11BL
MouseZYG11A
HumanZYG11A
MouseZYG11B
HumanZYG11B
zif1
zer1
99
99
99
89
94
66
85
58
66
88
81
59

Figure 2.11

Gastrulation defective mutants and RNAi, that do not make good candidates for a sensitized screen. Bar graphs representing the tabulated gastrulation defects in mutant alleles and injected dsRNAs, both internalization failure, where the E cells remain on the surface of the embryo (dark blue), and late internalization, where the E cells divide on the surface and internalize as 4 E cells (orange). Embryonic lethality is indicated on right. N values are found to the right of the bar graphs.

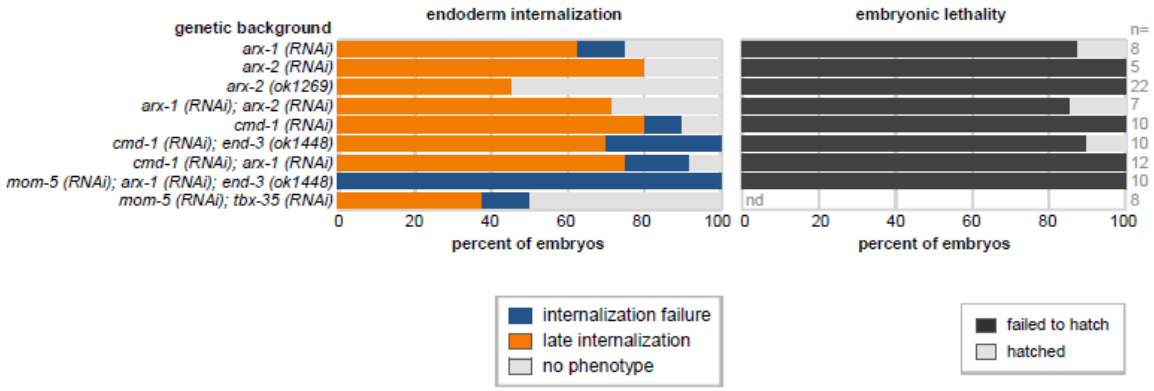


Figure 2.12

Primary screen feeding dsRNAs targeting 112 *sdz* genes into the gastrulation-sensitized background. Raw lethality of bacteria expressing dsRNA into wild-type (red) and gastrulation sensitized backgrounds, *ced-5(n1812);end-3(ok1448)*(blue). Results without error bars were not done in triplicate.


0
10
20
30
40
50
60
70
80
90
100
L4440-gfp
Par-6
gad-1
mom-5
hmr-1
acp-2
acp-5
alh-12
alh-13
apy-1
B0222.9
bro-1
C09B8.5
C10A4.5
C10C5.3
C17G1.7
C23H4.6
C33A12.12
C49C3.8
ckc-1
dhs-19
dhs-4
drr-1
end-1
end-3
F07A11.5
F12F6.8
F21C10.10
F28C6.10
F36A2.3
F37C4.6
F44A2.7
F47D12.5
F52H3.5
F53A2.3
F53H8.3
F55A4.8
F55B11.1
F58D2.1
fbxb-116
fbxb-119
fbxb-19
fbxb-35
fbxb-38
fbxb-61/F55C9.11
fbxb-83
fbxb-84
fipr-15
ftn-2
glo-3
hex-1
hgo-1
hlh-3
K04C1.3
K05F6.4
K09A11.1
kin-33
kin-34
nep-1
nhr-232
nit-1
nlp-29
prx-5
R07B7.10
R09H10.3/ZK697.8
sdz-1
sdz-10
sdz-11
sdz-13
sdz-15
sdz-18
sdz-19
sdz-2
sdz-20
sdz-21
sdz-22
sdz-23
sdz-24
sdz-25
sdz-27
sdz-28
sdz-29
sdz-3
sdz-31
sdz-32
sdz-33
sdz-34
sdz-35
sdz-36
sdz-38
sdz-4
sdz-5
sdz-6
sdz-7
sdz-8
sdz-9
set-10
T01H10.8
T01H8.2
T02H6.5
T22D1.11
T24C12.3
T26E3.8
T28C6.3
tag-120
tbx-11
tbx-35
twk-25
ugt-23
vet-6
W04A8.3
Y106G6H.4
Y116F11A.4
ZC376.3
ZK185.2
ZK688.7
N2
ced-5;end-3

Figure 2.13

Raw lethality resulting from feeding bacteria expressing dsRNA into wild-type and into single components of sensitized background. Percentage of lethality in *ced-5(n1812)* (red) and wild-type lethality (blue). Percentage of lethality in *end-3(ok1448)*(purple) and wild-type lethality (green). Error bars indicate 1 SE.

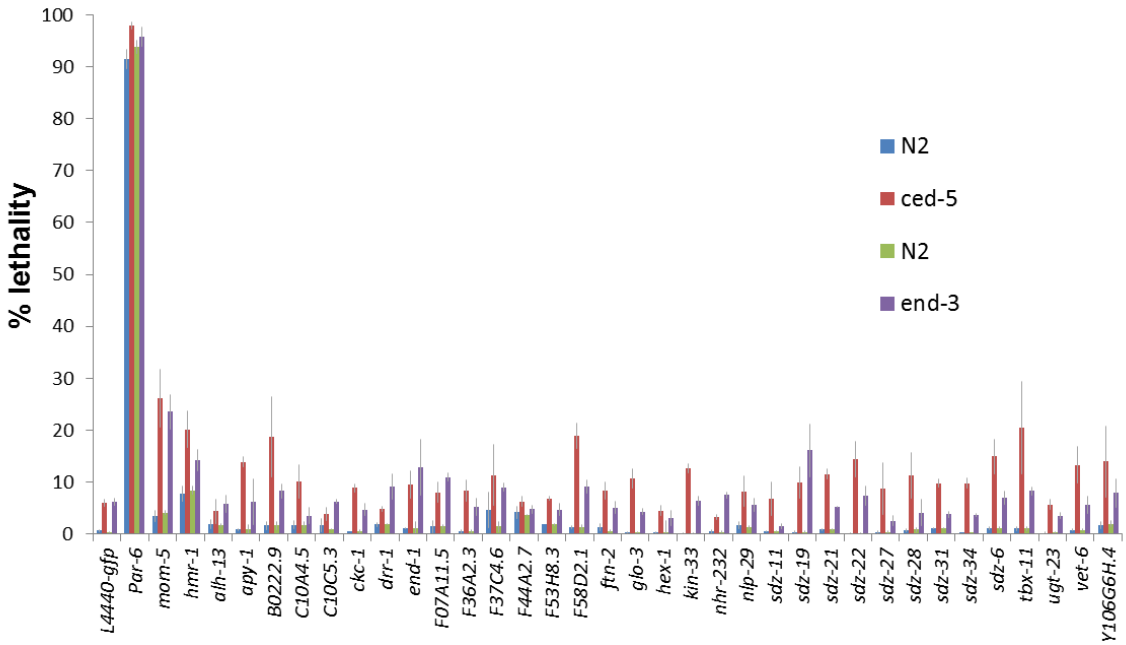


Figure 2.14

Relationship of *vhl-1*, *zif-1*, *zer-1*, and *zyg-11*, and human and mouse zyg11 homologs. This phylogeny show that the relationships among these genes using the trimmed conserved sequence alignment was consistent with previous analyses.

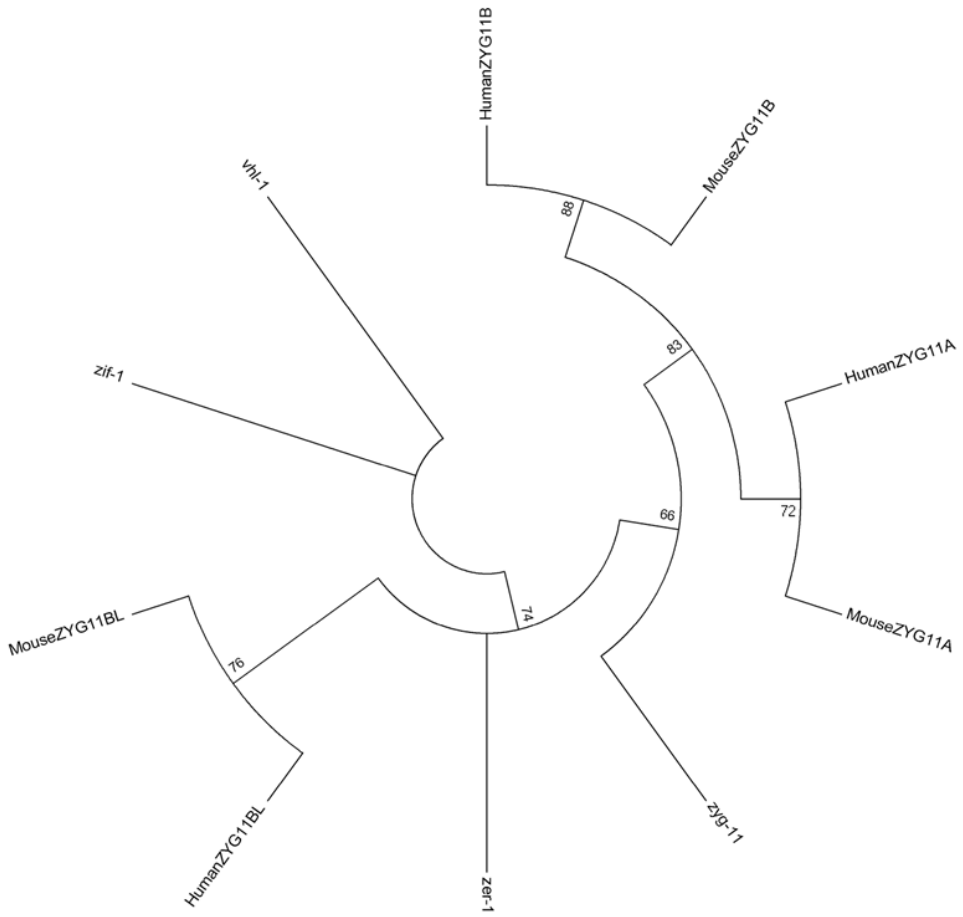


Figure 2.15

Maximum likelihood tree using full sequences of the newly identified genes and *vhl-1* as an outgroup. This tree shows that including the non-conserved sequences does not improve the phylogenetic relationships among the newly identified genes.

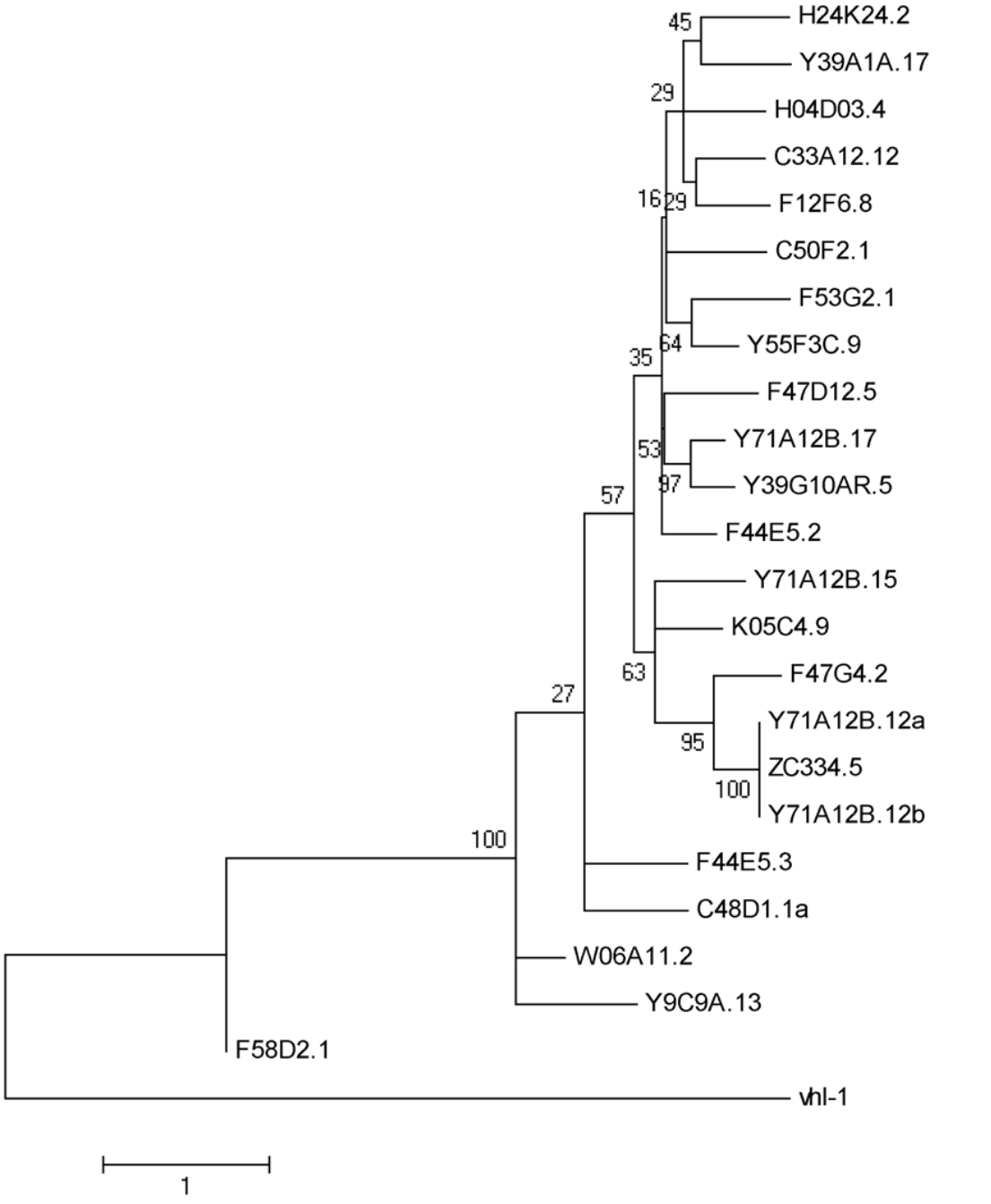

H24K24.2
Y39A1A.17
H04D03.4
C33A12.12
F12F6.8
C50F2.1
F53G2.1
Y55F3C.9
F47D12.5
Y71A12B.17
Y39G10AR.5
F44E5.2
Y71A12B.15
K05C4.9
F47G4.2
Y71A12B.12a
ZC334.5
Y71A12B.12b
F44E5.3
C48D1.1a
W06A11.2
Y9C9A.13
F58D2.1
vhl-1
45
29
16
29
64
35
53
97
57
63
27
95
100
100
1

Figure 2.16

GADR-5/Y71A12B.17 and GADR-6/F47G4.2 physically interact with both CUL-2 and ELC-1 when co-expressed in human cells. FLAG-tagged GADR-5, GADR-6, and ZYG-11 were co-expressed in HEK293T cells with CUL-2-Myc or HA-ELC-1 as noted by (+) symbols above the lanes. Anti-FLAG immunoprecipitations (IP) and lysates were analyzed by western blot using anti-FLAG, anti-HA, or anti-CUL-2 antibodies. A cross-reacting band serves as a loading control. Note that both GADR-5 and GADR-6 bind CUL-2 and ELC-1 analogous to the known substrate recognition subunit ZYG-11. The smearing and additional lower bands for FLAG-GADR-5 presumably arise from partial degradation of the protein in HEK293T cells. (*) denotes the heavy chain of IgG used in the IP; (**) marks non-specific band (which co-migrates with lower band of CUL-2 in the first four samples).

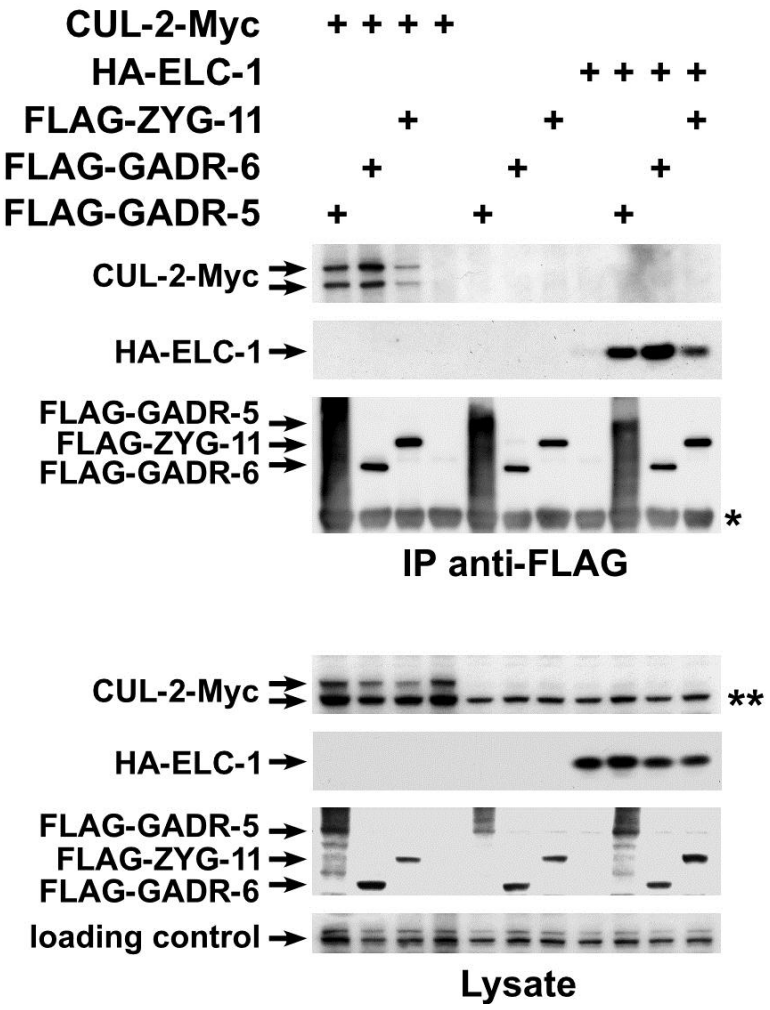

CUL-2-Myc + + + +
HA-ELC-1 + + + +
FLAG-ZYG-11 + + +
FLAG-GADR-6 + + +
FLAG-GADR-5 + + +
CUL-2-Myc
HA-ELC-1
FLAG-GADR-5
FLAG-ZYG-11
FLAG-GADR-6
*
IP anti-FLAG
CUL-2-Myc
**
HA-ELC-1
FLAG-GADR-5
FLAG-ZYG-11
FLAG-GADR-6
loading control
Lysate

Table 1

F58D2.1 defines a new gastrulation gene family in *C. elegans.* Injection of dsRNAs targeting individual family members into the sensitized background, *ced-5(n1812)*, showed that multiple paralogs function in gastrulation (left). However, using dsRNA to individually target these genes in wild-type did not cause any apparent gastrulation defects (right). Colored genes indicate the genes used in Table 2.

	ced-5			**N2 (wildtype)**		
Gene	**Gastrulation Defects (n)**	**Total (n)**	**Gastrulation Defects (%)**	**Gastrulation Defects (n)**	**Total (n)**	**Gastrulation Defects (%)**
F58D2.1	8	32	25	0	28	0
C48D1.1A	3	14	21	0	15	0
C33A12.12	3	15	20	0	12	0
F53G2.1	4	18	22	0	20	0
F12F6.8	2	24	8	0	18	0
F47D12.5	4	24	17	0	18	0
W06A11.2	4	34	12	0	15	0
Y71A12B.17A	2	6	33	0	26	0
zeel-1	0	13	0	nd	nd	nd
F47G4.2	2	7	29	0	13	0

Table 2

F58D2.1 and paralogs are additive, redundant gastrulation genes. The six with the most specific effects on gastrulation were targeted in a pool, by injecting multiple dsRNAs. Sub-pooling was achieved by removing the gene not found within the pool that gave the greatest percentage gastrulation defects (see Results). Genes are color-coded for ease of following the pools.

Genes in Pool						Gastrulation Defects (n)	Total (n)	Gastrulation Defects (%)
F58D2.1	*C33A12.12*	*F47D12.5*	*W06A11.2*	*Y71A12B.17A*	*F47G4.2*	27	55	49
	C33A12.12	*F47D12.5*	*W06A11.2*	*Y71A12B.17A*	*F47G4.2*	7	51	14
F58D2.1		*F47D12.5*	*W06A11.2*	*Y71A12B.17A*	*F47G4.2*	7	30	23
F58D2.1	*C33A12.12*		*W06A11.2*	*Y71A12B.17A*	*F47G4.2*	3	12	25
F58D2.1	*C33A12.12*	*F47D12.5*		*Y71A12B.17A*	*F47G4.2*	6	18	33
F58D2.1	*C33A12.12*	*F47D12.5*	*W06A11.2*		*F47G4.2*	8	51	16
F58D2.1	*C33A12.12*	*F47D12.5*	*W06A11.2*	*Y71A12B.17A*		14	39	36
	C33A12.12	*F47D12.5*	*W06A11.2*	*Y71A12B.17A*		2	21	10
F58D2.1		*F47D12.5*	*W06A11.2*	*Y71A12B.17A*		2	38	5
F58D2.1	*C33A12.12*		*W06A11.2*	*Y71A12B.17A*		28	88	32
F58D2.1	*C33A12.12*	*F47D12.5*		*Y71A12B.17A*		5	47	11
F58D2.1	*C33A12.12*	*F47D12.5*	*W06A11.2*			6	43	14
	C33A12.12		*W06A11.2*	*Y71A12B.17A*		6	44	14
F58D2.1			*W06A11.2*	*Y71A12B.17A*		24	68	35
F58D2.1	*C33A12.12*			*Y71A12B.17A*		1	17	6
F58D2.1	*C33A12.12*		*W06A11.2*			3	23	13
			W06A11.2	*Y71A12B.17A*		2	19	11
F58D2.1				*Y71A12B.17A*		8	45	18
F58D2.1			*W06A11.2*			2	17	12

Table 3

New gastrulation genes from gastrulation-sensitized screen, RNAi-sensitized screen, and mutant alleles. E cell fate was perturbed in embryos with gastrulation defects from three of these genes (on right). Sixteen non-redundant genes are listed before the 13 redundant genes.

Gene	**description**	**No $P_{end\text{-}1}$::GFP**
acp-2	acid phosphatase	**1/1**
apy-1	Apyrase	0/5
B0222.9	xanthine dehydrogenase	n/a
C10A4.5	Unknown (4 transmembrane domains)	**2/6**
glo-3	Gut granule/lysome formation	0/2
kin-33	Kinase domain	0/1
sdz-6	Unknown	0/2
sdz-19	Unknown	n/a
sdz-22	Transthyretin-like	n/a
sdz-27	Unknown	0/1
sdz-28	BTB/POZ domain	0/8
sdz-31	Hemocyanin, copper-containing	0/1
sdz-36	Unknown	0/2
tbx-11	T-box Transcription factor	n/a
ugt-23	Glycosyltransferase family 28	**1/1**
vet-6	Unknown (very early transcript)	0/2
alh-13	Amino acid kinase family	n/a
drr-1	Unknown	n/a
F44A2.7	Unknown	n/a
F58D2.1	ZYG-11 protein like	n/a
fbxb-19	F-box protein	n/a
fbxb-35	F-box protein	n/a
fbxb-38	F-box protein	n/a
prx-5	Peroxisomal-like protein	n/a
sdz-18	Unknown	n/a
sdz-21	Unknown	n/a
sdz-23	EGF domain	n/a
sdz-32	Unknown	n/a
sdz-34	Zinc RING finger	n/a

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CHAPTER 3

Discussion and Future Experiments

Morphogenesis, or how cells and tissues become organized in the developing organism, remains an outstanding problem within the field of developmental biology. The link between cell fate specification and the mechanisms of cell movements is not well understood. Apical constriction continues to be a leading example in our understanding of these connections (reviewed in Chapter 1). However, a major hurdle to the study of morphogenesis is the proposed redundancy of genetic mechanisms involved. In Chapter 2, we addressed this proposed problem, describing my efforts to find new genes that affect the movement of E cells during *C. elegans* gastrulation. In this section, I will expand on future questions and experiments that were introduced in Chapters 1 and 2.

On the nature of screens.

In Chapter 2, we described our reasoning for doing an RNAi enhancer screen. Historically, enhancer screens have been used to elucidate several pathways, most famously the receptor tyrosine kinase pathway (Sevenless) in *Drosophila* (reviewed in Simon, 1994; St Johnston, 2002). By using an already deficient mutant for a pathway, enhancers and suppressors of that pathway can be found by making that sensitized background more or less defective, respectively. Besides elucidation of a pathway,

another point worth noting from these screens was the number of false positives that modified the phenotype by enhancing an unrelated process. These indirect effects are a major disadvantage to the methodology of enhancer screens, or as Dave Reiner would say, "How do you know you aren't just making them sicker?" Although, in Chapter 2 we did find genes involved directly in *C. elegans* gastrulation, further pathway analysis with null mutants remains future work to determine what processes are being perturbed to disrupt gastrulation.

Mutant analysis is not the only manner to accomplish an enhancer screen. Kirschner and others have taken great advantage of introducing RNAs directly into embryos to enhance or suppress phenotypes to screen for proteins in several pathways, including Noggin and Nodal signaling (e.g., Lustig and Kirschner, 1995; Lustig *et al*., 1996; Kroll *et al*., 1998). Although these direct expression screens were fundamentally different than the RNAi enhancer screen we pursued, it demonstrated one of the major advantages in these screening methods. Once a positive RNA demonstrates the desired result, the sequence of the RNA injected is in hand and no further positional cloning is required.

Within *C. elegans*, there have been several modifier screens (Labbé et al., 2006; O'Rourke et al., 2007; Dorfman et al., 2009), as mentioned in Chapter 2, but there is also an enhancer screen for morphogenesis proteins using a sensitized background, by Abby Cox and Jeff Hardin, however their screen is unpublished. By using a hypomorphic allele to alpha-catenin, *fe4*, they were able to enhance for ventral enclosure mutants in developing embryos. We used a similar strategy in our screen, with one major exception;

we enhanced the defects in null mutants with subtle gastrulation phenotypes, not a weak allele.

With this historical perspective in mind, how does one set up a screen to find new genes required for gastrulation in *C. elegans*? Again, in Chapter 2, we detailed our reasoning for an RNAi enhancer screen, but specifically we selected for the screen candidate genes previously indicated to be transcribed in endo-mesodermal cells, but there are many other candidates that could be explored in a gastrulation-sensitized background. A post-doc in the lab, Jessica Sullivan-Brown, is doing just that. Her candidates are genes with known roles in neural tube closure in vertebrates and genes implicated in the human disease *spina bifida*. Positives from this screen may suggest evolutionarily conserved mechanisms of morphogenesis, specifically those using apical constriction. Other candidates tested by me and others in the lab for gastrulation defects are all the known actin regulators and interactors. Many appeared to have no effect on gastrulation when tested individually. With our new understanding of the prevalence of redundancy, these genes should be re-tested in the context of the sensitized screen. Similarly, in light of preliminary data of a former post-doc, Dan Marston, that some worm cadherin family members have a redundant gastrulation phenotype, adhesion molecules in *C. elegans* should be retested in sensitized backgrounds.

Why not try to get a genome-wide view? By doing a genome-wide, non-candidate approach to the screen, we may identify groups of genes we would not have predicted based on our current knowledge. A standard forward mutagenesis screen at this point is not ideal because 1) we do not have an easy method to screen through first-pass phenotypes besides lethality and 2) cloning of mutants, although faster than in years past,

is still not ideal. A genome-wide 96-well liquid culture feeding RNAi screen (Ahringer, 2006), however, is very possible, since we have demonstrated that lethality can be enhanced. Using liquid culture for an RNAi screen and employing automated techniques make this possibility even more feasible, with a COPAS biosorter used for both the "front-end" loading of the worms into 96 well plates and the "back-end" for scoring analysis (Furlong et al., 2001), as is described in more detail below.

To streamline this automated screen both the sensitized worms and the control (non-sensitized) worms can be placed into the same wells containing the test dsRNA. This control adds the constraint that both sets of worms are under the exact same conditions. So, on the "front-end" of the experiment, the biosorter adds 4 of the sensitized L4 worms and 4 of the L4 control worms into each of the 96 wells containing test and control dsRNAs. Either the sensitized or the control worms would have a larval-expressed GFP to distinguish the two backgrounds. For instance, $_{p}$*myo-2*::GFP could be used since this marker begins fluorescing early and remains bright throughout the worm's life, for easy detection. The other worms could have a separate marker in another color, such as red, and the embryos could have a separate color, for instance cyan, but neither is explicitly necessary.

After 48 hours, when the worms digesting the bacterially-expressed dsRNA have laid all their eggs, their resultant offspring are counted. The biosorter then helps on the "back-end" of the experiment by counting the worms in each well based on length and whether they are positive for the fluorescent marker of choice. Defect-enhancing bacterially expressed dsRNAs will affect the ratios of resultant GFP positive to negative worms counted by the acquiring software. For instance, when the sensitized strain was

marked with the GFP, if a bacterially-expressed dsRNA enhanced lethality in the sensitized strain, then the number of non-GFP offspring would be considerably higher than the GFP-positive worms. Embryos could also be counted or even differentiated between worm strains if a third color was used, but this would serve more as a double check than a necessity. Even though the biosorter takes some time to run per plate, it saves time by counting the controls and experiment at the same time. In a replicate run-through, alternate markers could be used to filter out false positives due to the specific fluorescent worm strains used. Positive hits for this primary screen could be verified by our feeding methods on worm plates, or directly taken to a secondary screen, such as by injection of dsRNA and 4-D microscopy.

Both candidate and genome-wide screens create lists of genes that contribute to a biological process. Ideally, some of the proteins that these genes encode group together logically or are even part of the same complex. Grouping these genes is significantly more direct if all the players are in hand. In morphogenesis, the difficulty in getting a handle on many of the players has been claimed to be due to the challenge of redundancy. Our work has addressed that challenge and added to the fold some these often more difficult to identify genes.

The genes themselves.

The screen described in Chapter 2 found many genes with partial and subtle defects in gastrulation, but what roles do these genes actually play in gastrulation? Specifically, do they have direct roles in apical constriction or non-specific roles that indirectly affect gastrulation? Which of the new positive gastrulation genes are redundant

with each other? By crossing the null alleles that demonstrate gastrulation defects and/or using RNAi of these positive gastrulation genes into those null alleles, the question of redundancy could easily be addressed using classic epistasis experiments to delineate pathways. Perhaps a sensitized strain that enhances only apical constriction defects, or enhances other specific contributing gastrulation processes, could be obtained.

How do non-homologous genes in vastly different cellular processes aid in the overall morphogenesis? We have presented in Chapter 2 a story on redundancy and hinted at the possibility of the evolutionary expansion of genes in a crucial morphogenetic process, gastrulation, but there is also the possibility for genes aiding in the process of gastrulation very indirectly. These sorts of non-linear redundancies are similar to concepts of distributed robustness, as hypothesized about *C. elegans* vulval development (Felix and Wagner, 2008). In short, no two proteins need to have the same function, for a resulting enzyme or biochemical substrate to be made. In this way, an end result is achieved without any direct substitution, as we have stated before, a non-homologous redundancy. However, they propose that this sort of system aids in robustness, especially in development stages to account for different environments and perturbations. Sorting through the genes that affect gastrulation via processes outside of apical constriction might help determine the sorts of cell mechanisms that aid in the robustness of the early development of *C. elegans*, specifically the initiation of gastrulation.

What about genes that do directly affect apical constriction? Our lab has begun to analyze myosin and actin movements in the E cells and surrounding cells prior to and during E cell internalization. Others in our lab have found that when *hmr-1* and *ced-5* are

both depleted, constantly flowing myosin continues to move, even though the membranes at the E cell borders do not (Roh-Johnson et al., unpublished). The screen in Chapter 2 has found 29 new genes with enhanced roles for gastrulation in the *ced-5* background. Do their gastrulation defects phenocopy *hmr-1*? Careful high-speed ventral view movies will need to be assessed for these gastrulation genes to determine which of them also maintain proper acto-myosin dynamics during apical constriction.

GADR-1 to -6

As introduced in Chapter 2, GADR-1 to -6 are most similar to the *C elegans* protein ZYG-11. ZYG-11 is the substrate recognition subunit (SRS) of a Cullin-2 (CUL-2) ubiquitin ligase degradation system (Vasudevan et al., 2007). The mutant phenotypes of *cul-2* include a wide range of perturbed processes in *C. elegans*, including cell-cycle defects, polarity defects including the ectopic localization of PAR-2, ectopic cytoplasmic extensions, and meiotic defects, however even within the very early embryo not all of the phenotypes of *cul-2* can be explained by the known *C. elegans* SRS genes, *zyg-11*, *zif-1*, *zer-1*, and *vhl-1* (DeRenzo et al., 2003; Feng et al., 1999; Kemphues et al., 1986; Liu et al., 2004; Sonneville and Gonczy, 2004; Vasudevan et al., 2007). Taken together, this suggests CUL-2 may interact with other SRS proteins and these interactions may explain the remaining known, and unknown, CUL-2 phenotypes.

I have begun to test the function of the putative SRS's, *gadr-1* to *-6*. Preliminary data indicated that when *gadr-1* to *-6* are simultaneously depleted by RNAi, myosin and apical PAR accumulation and localization appear normal (Figure 3.1 A-H). The basolateral PAR-2 localized correctly, but also may accumulate a cytoplasmic pool

(Figure 3.1 I,J). These data suggest that for the *gadr-1* to *-6*-depleted embryos, we can rule out a few protein's accumulation and localization as likely explanations for gastrulation defects, however PAR-2 cytoplasmic accumulation warrants further quantification.

Interestingly, while I was assessing the PAR-2:GFP movies, I observed large, dark (GFP-absent) vesicles moving about in the cytoplasm, that I had not seen in the non-injected PAR-2:GFP embryos. I had also noticed these large vesicle-like holes in fixed embryos (Figure 3.1 F), but had thought they were the product of fixation conditions. I have attempted to identify these dark spots by using an antibody toolkit developed to test vesicle type (in parenthesis): CAV-1 (caveolae), CYP-33a (endoplasmic reticulum), SQV-8 (Golgi), LMP-1 (lysosomes), RME-1 (recycling endosomes), DYN-1 (sites of clathrin-mediated endocytosis), APA-2 (clathrin adaptor) (Hadwiger et al., 2010). So far, I could not detect a difference in DYN-1 or RME-1 immuno-staining between wildtype embryos and those depleted with the pool of *gadr-1* to *-6*. However, I did see an increase in LMP-1-positive spots in the dsRNA injected embryos (n=1). Additional experiments and careful analysis of dsRNA-injected control embryos will need to be done to determine if the vesicle accumulation in the mutants are actually lysosomes. If they are lysosomes, than these results would be supportive of the hypothesis that GADR-1 to -6 are involved in degradation.

In *C. elegans*, the von Hippel-Lindau (VHL) box of SRS proteins has been shown to be required for binding to Elongin-c, ELC-1 (Vasudevan et al., 2007). With the recent finding that two of these family members, GADR-5 and GADR-6, bind CUL-2 and ELC-1 by CO-IP (Figure 2.16), we hypothesize that these paralogs are specifically the SRS

proteins of a CUL-2 degradation system. If so, what do they degrade? Given their homology with zyg-11, the targets of GADR-1 to -6 would presumably be proteins that must be ubiquitinated for proper gastrulation to occur. Previous proposals of doing mass spectrophotometry to identify interactors of our potential SRS proteins using the antibodies we have generated will be pursued further by our collaborators (Kipreos, personal communication).

END-1 and END-3

One trend that has come out of the generation of the sensitized strain and the secondary screening is that *end-3* and *end-1* are not equivalent or completely redundant transcription factors (Maduro et al., 2005), nor do they both have equal gastrulation defects (Lee et al., 2006). The gastrulation sensitized strain, *ced-5;end-3*, did not exhibit enhanced lethality or enhance the gastrulation defects relative to either single null allele (Chapter 2). Likewise, both *mom-5* and *hmr-1* enhanced each of these null alleles separately. Are *mom-5*, *hmr-1*, and *end-1* epistatic to each other? What is upstream of what? How do the new genes found in this screen fit into this picture? Teasing apart this pathway could be very informative to clearly defining exactly how cell fate is properly linked to apical constriction during gastrulation.

Low hanging fruit.

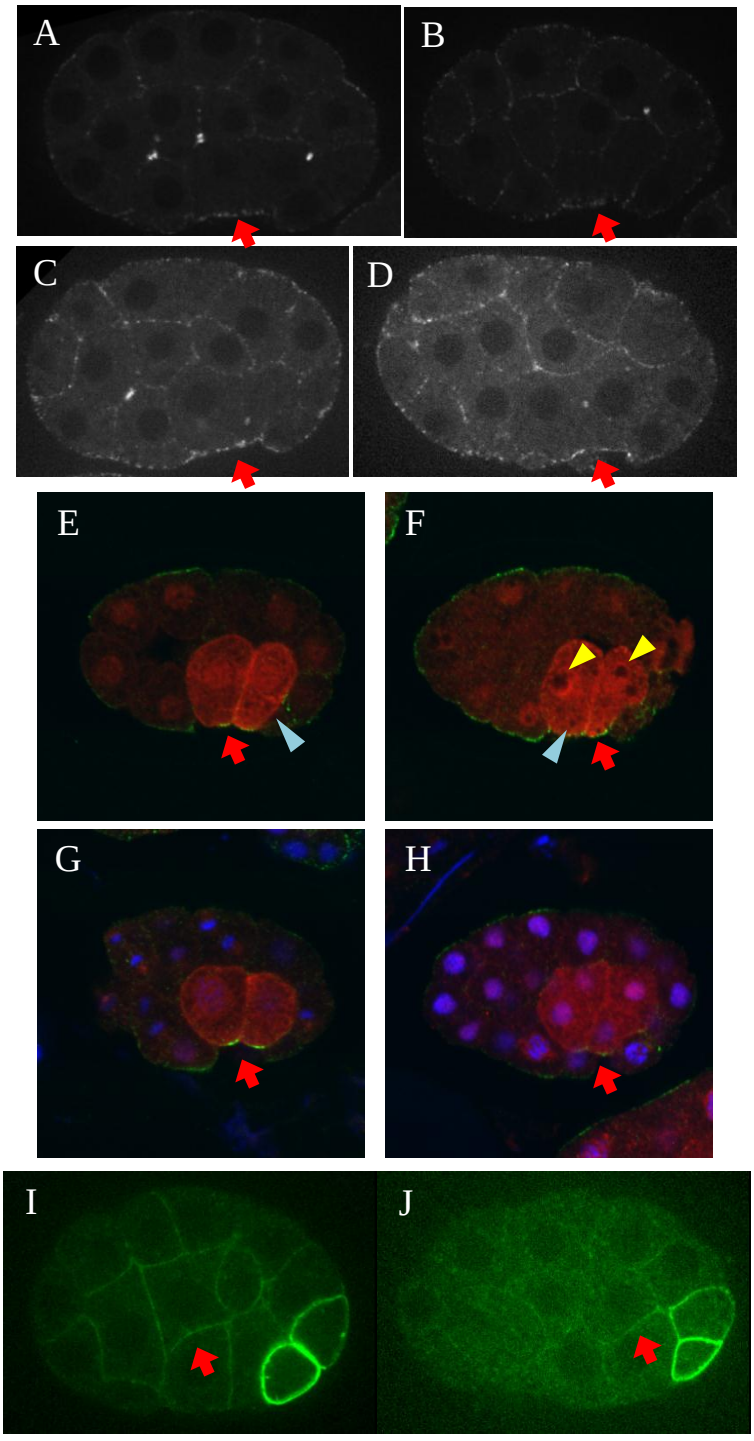
As any area of scientific research is formed, the first advances are nearly always the most easily attacked first, the low hanging fruit. A major open question in developmental biology is the link between cell fate specification and mechanisms of cell movements. A major hurdle to this question is the redundancy of genetic mechanisms

involved. Previous attempts to identify genes involved in gastrulation, in diverse organisms, have only identified a small set of genes (Wieschaus 1995). We also found that feeding individual dsRNAs has yielded few new genes for gastrulation and embryonic development (data not shown). Currently, many of the genes where a single mutation gives an exciting phenotype are being studied. If we want to get a handle on all the players, then the time has come to get a handle on proteins having redundant and subtle roles in development and morphogenesis. *C. elegans* is an excellent model system for resolving problems of redundancy, since several techniques allow one to simultaneously disrupt the function of multiple genes at once. I have used three strategies to attack this problem: 1) creating double (and triple) mutants of the known players, 2) RNAi (feeding or injection) into genetically sensitized backgrounds, and 3) pooling dsRNAs to deliver by injection, targeting multiple candidate genes. Although this work is focused on *C. elegans* gastrulation, it is also an attempt to tackle the problem of redundancy and expand the field to face this challenge head-on, reaching higher into the tree.

FIGURES

Figure 3.1

Non-muscle Myosin, PAR-3, and PAR-2 in *gadr-1* to *6* depleted embryos. Non-injected embryos (A,C,E,G,I) vs. embryos injected with pools of 6 dsRNA for *gadr-1* to -*6* (B,D,F,H,J). (A,B,C,D) Ventral images from NMY-2:GFP movies. Red arrows indicate proper accumulation and localization of myosin. (E,F,G,H) Immunohistochemistry of gastrulation stage embryos 26-28 cells with PAR-3 (green), E cells (red), and DAPI (blue), just prior to E cell ingression (E,F) and during ingression (G,H). The depletion of *gadr-1* to -*6* by RNAi does not seem to deplete PAR-3 accumulation or localization (red arrows). Large, intriguing dark spots can be seen in F (yellow arrowheads). Small dark spots can be seen both in injected or non-injected embryos (blue arrowheads). (I,J) Ventral images from PAR-2::GFP movies. PAR-2 appears to properly accumulate and localize to the basolateral membrane (red arrows), but the cytoplasmic pool of PAR-2::GFP appears much higher in the *gadr-1* to -*6* embryos. Scale: *C. elegans* embryos are approximately 50µm long.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